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Interactions of *Teladorsagia circumcincta* with the ovine immune system – mimicry and vaccine development

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Declaration

The work presented in this thesis is my own work, unless otherwise stated, and has not been submitted for any other degree.

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Abstract

Teladorsagia circumcincta, an economically-important abomasal nematode of small ruminants in temperate regions worldwide, is currently controlled with a combination of anthelmintics and pasture management. Anthelmintic resistance has emerged and vaccination is a potential alternative control strategy, as protective immunity in sheep can be acquired after repeated exposure to the parasite. Abomasal mucosal IgA responses in immune sheep have been correlated with delayed worm development and reduced faecal egg counts. However, recombinant vaccine development against parasitic nematodes has had limited success, and one of the reasons may be unsuitable expression systems for antigen production leading to incomplete or inadequate post-translational modifications such as glycosylation and tertiary protein folding, resulting in incorrect epitope structures for antibody binding. In this thesis, to address this issue, "native" infective larval (L_3) antigen targets of protective immune responses and synthetic peptide sequences which mimic structural epitopes on these antigens were identified. Abomasal mucosal IgA was used as a probe to identify native immunogenic antigens from *T. circumcincta* L_3. IgA was purified from abomasal mucus of animals rendered immune by repeated experimental infection and a custom antibody-affinity column was created and used to purify antigens from an L_3 somatic PBS-soluble extract. Affinity purified L_3-antigen-specific IgA levels in sheep with varying levels of immunity to *T. circumcincta* were positively correlated ($r_s = 0.853$, $P < 0.001$) with both the total IgA concentration in efferent gastric lymph after parasite challenge, and with the percentage of inhibited fourth-stage (L_4) larvae present in the gastric glands of the immune hosts ($r_s = 0.534$, $P = 0.007$). In contrast, a negative correlation between the levels of affinity-purified L_3 antigen-specific IgA and total *T. circumcincta* burden was observed ($r_s = -0.565$, $P = 0.004$). Proteomic analysis of the IgA-affinity purified L_3 extract identified a number of proteins which represent potential vaccine candidate molecules in other helminth species, including paramyosin, superoxide dismutase, galectin, activation-associated secreted proteins and fatty-acid retinol-binding proteins. As a first step towards the development of a novel vaccine based on IgA-binding peptide mimics of native structural epitopes, phage display libraries were used to screen

antibodies, from sheep rendered immune to *T. circumcincta* by experimental infection. These antibodies were affinity-purified before use and specifically bound *T. circumcincta* L_3 glycans or, alternatively, surface antigens on exsheathed *T. circumcincta* L_3. Five peptide sequences which mimic L_3 antigenic epitopes were identified and positive correlations existed between peptide-specific IgA levels and both the total IgA concentration in efferent gastric lymph after parasite challenge and the percentage of inhibited L_4 present ($r_s > 0.621$, $P < 0.001$ to $P < 0.05$). In contrast, negative correlations between the levels of peptide-specific IgA and the total nematode burden were observed ($r_s > -0.528$, $P < 0.01$ to $P < 0.05$). In conclusion, the selected phage clones may therefore represent vaccine candidates if they could be presented to the ovine immune system in an appropriate fashion.

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Chapter 1 : General Introduction

1.1 Parasitic gastroenteritis

Parasitic nematodes are a serious issue affecting livestock agriculture due to the economic costs associated with infection and the impact upon welfare. In small ruminants, helminth infections are one of the most important causes of production losses worldwide (Molento, 2009). Parasitic gastroenteritis (PGE) is caused by the presence of parasitic nematodes in the gastrointestinal tract and can substantially affect lamb productivity due to poor protein and fat deposition, anaemia, intermittent diarrhoea leading to weight loss or less than optimal live-weight gain (Coop and Kyriazakis, 2001; Sargison, Scott and Jackson, 2002; Taylor, Coop and Wall, 2007). Sub-clinical infections are associated with a suppression in appetite (Greer *et al*., 2008; Miller and Horohov, 2005), which can lead to a low live-weight gain and poor body condition (Taylor, Coop and Wall, 2007). Previously, it was estimated that gastrointestinal nematode infections cost the UK sheep farming industry in excess of £84 million per annum (Nieuwhof and Bishop, 2005). These figures include costs due to poor lamb productivity and those associated with the treatment of infections; however, they exclude the costs of sub-clinical infections.

1.1.1 *Teladorsagia circumcincta*

Teladorsagia (Ostertagia) circumcincta, commonly known as the 'brown stomach worm', is a highly prevalent parasitic nematode of sheep in temperate regions and is the most important cause of PGE in these areas (Sargison, Scott and Jackson 2002; Taylor, Coop and Wall, 2007): *T. circumcincta* has been reported as the most prevalent gastrointestinal nematode in small ruminants in the UK (Bartley *et al*., 2003). *T. circumcincta* belongs to the order Strongylida and is a member of the superfamily Trichostrongyloidea, along with other small ruminant nematodes; *Haemonchus*

contortus, *Cooperia* spp., *Nematodirus* spp. and *Trichostrongylus* spp. (Durette-Desset *et al*., 1999; Taylor, Coop and Wall, 2007).

1.1.2 Life cycle

T. circumcincta has a direct life cycle (Figure 1.1) with a definitive ruminant host, similar to all members of the Trichostrongyloidea family. Eggs are released by the adult females that inhabit the abomasum, pass through the gastrointestinal tract and are deposited onto pasture in the faeces. If conditions are favourable for survival, eggs hatch and develop into the first larval stage (L_1) in 24 hours and then feed on bacteria present in the faeces. Next, L_1 undergo a first larval moult into second-stage larvae (L_2), which also feed on bacteria. Approximately 10-14 days later, the L_2 moult to the third larval stage (L_3) and the L_2 cuticle is retained as a protective sheath; however, this prevents feeding (Keith *et al*., 1990). Under optimal conditions L_3 migrate from faeces onto pasture. Optimal temperature and humidity ranges for *T. circumcincta* larval development are 18-26°C and 60% humidity (O'Connor, Walkden-Brown, and Kahn, 2006; Urquhart *et al.*, 1996). In recent years, autumn and winter seasons have been milder and wetter, which means that average temperatures persist at 10°C for a longer period of the year, allowing more larvae to develop on pasture (Kenyon *et al.*, 2009; Van Dijk *et al.*, 2010). *T. circumcincta* L_3 have been reported to be able to survive for up to 13 weeks at -10°C (Pandley *et al*., 1993). Infective L_3 are ingested by the ruminant host, they exsheath in the ruminoreticulum and migrate to the abomasum where they can be found in the gastric glands approximately 24 – 48 hours post-infection (Michel, 1974). Following establishment in the abomasum, L_3 continue development through another two moults to the fourth stage larvae (L_4) (approximately 5-7 days post-infection (dpi)) and then fifth stage larvae (L_5) (approximately 10-14 dpi) which are immature adult worms. L_4 emerge from the gastric glands onto the lumenal surface of the abomasum approximately 10 dpi. Female worms become sexually mature approximately 16-21 dpi,

with the complete life cycle of *T. circumcincta* from eggs to adults being completed in approximately 21 days (Soulsby, 1982; Urquhart *et al.*, 1996).

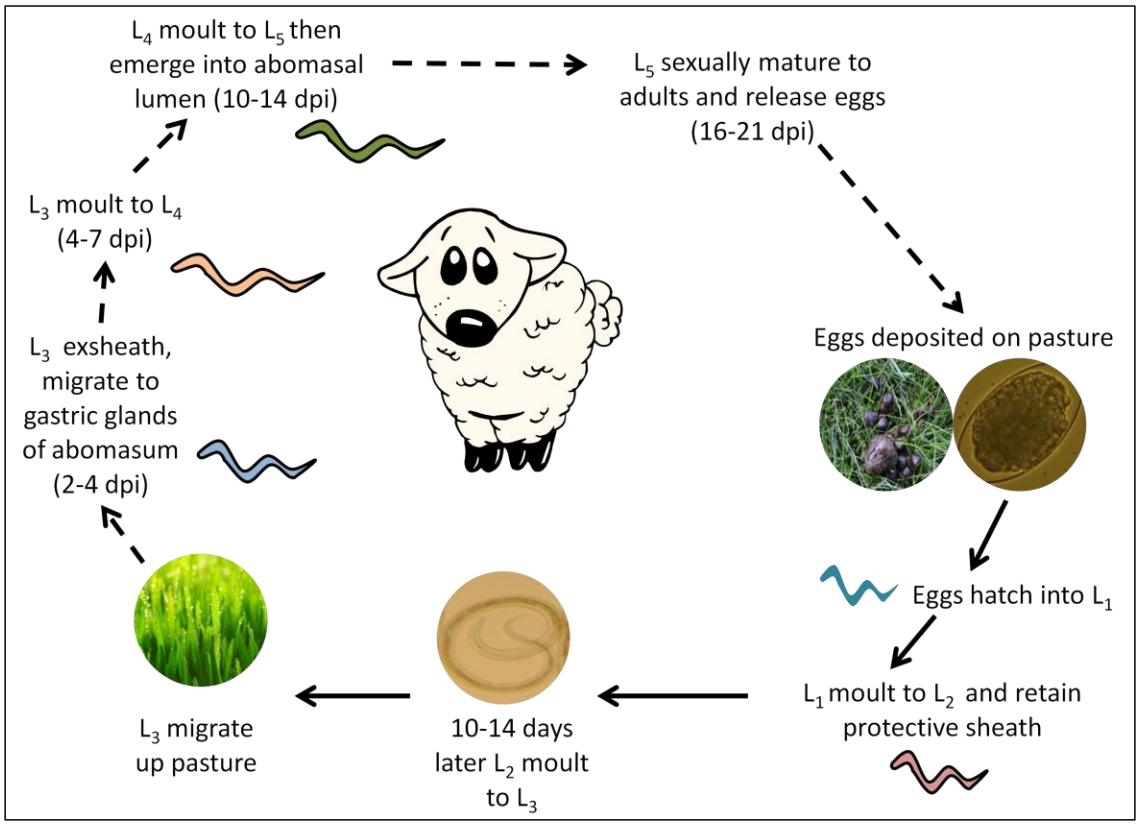

Figure 1.1 Schematic representation of the life cycle of *T. circumcincta*.

Images courtesy of D. McBean (Disease Control, Moredun Research Institute). Dashed lines represent parasitic stages which are either ingested or deposited by the host.

Under some circumstances, *T. circumcincta* L_4 enter a process known as arrested larval development in the gastric gland. Specifically, in *T. circumcincta,* studies have shown that larval development can be arrested in response to pressure from the ovine immune system (Smith, 2007). Arrested L_4 can reside in the abomasal glands for several months before resuming development through the life cycle (Michel, 1974). Repeated, prolonged exposure of the host to *T. circumcincta* can lead to protective immunity (see Section 1.3) but, during the lambing and lactation period, a phenomenon termed the 'peri-parturient relaxation' (PPR), leads to a temporary loss of immunity in ewes. This has been linked to competing protein demands in the host during late pregnancy and

lactation (Houdijk *et al*., 2001; McAnulty *et al*., 2001). The PPR in immunity and the associated reduction in serum IgA levels have been correlated with increased worm burdens in ewes, with subsequent increased egg contamination of pasture prior to turn-out of lambs (Houdijk *et al*., 2001; Jeffcoate *et al*., 1992). A third source of infection for lambs in the spring is from over-wintered infective L_3. Recently, cases of exceptionally high faecal egg counts in 6 month-old lambs have been reported (Kenyon *et al*., 2009). As worm burden is acquired over a number of weeks this indicates increased challenge on pasture, which could potentially be linked to milder, wetter winters allowing more larvae to overwinter on pasture.

1.2 Disease pathogenesis

The presence of adult nematodes on the surface of the abomasum and the emergence of developing L_4 from the gastric glands causes severe damage to the abomasum (Balic, Bowles and Meeusen, 2000). This results in an increased requirement for protein for tissue repair in the gastrointestinal tract. The nutritional cost to the host to initiate an immune response, combined with plasma protein leakage through mucosal epithelial cells, results in reduced protein metabolism (Greer *et al*., 2008; McKellar, 1993; Simpson, 2000).

During infection, abomasal mucosal damage is characterised by swollen gastric glands (Figure 1.2), cellular infiltration and cytolysis of the epithelial cells lining the luminal surface of the mucosa, resulting in loss of integrity and secretory function of the cells (Scott *et al*., 1998; Taylor, Coop and Wall, 2007). Loss of the normal secretory cells leads to protein-losing gastropathy, causes hypoalbuminaemia and impacts negatively upon the host's nutritional status (McKellar, 1993; Sutherland and Scott, 2010). At 5 dpi, coincident with L_4 emergence from glands, acid secretion from the abomasum is reduced and the pH of the abomasal contents increases, which in turn reduces pepsinogen activation and increases gastrin secretion (Lawton *et al*., 1996; Simpson, 2000).

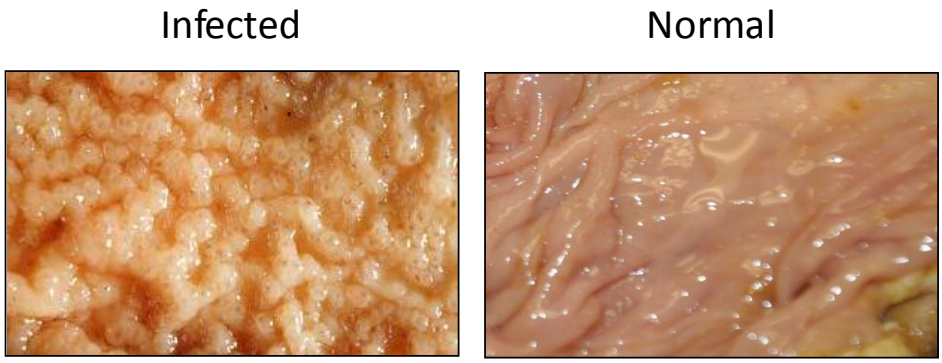

Figure 1.2 Images illustrating the pathological damage to an abomasum from a sheep during an infection with *T. circumcincta* compared to an abomaum from a parasite-free sheep.

The abomasum has a soft, mucous surface which is composed of longitudinal folds. The abomasum from a sheep during an infection with *T. circumcincta* characteristically has nodules ('polyps') present on the mucosal surface of the abomasums due to the presence of L_4 in the gastric glands. Images kindly provided by Dr D.J. Bartley, (Disease Control, Moredun Research Institute).

Changes in the physiology of the abomasum are also seen; mucus production is increased, pepsin secretion is inhibited, and gastrin levels in the serum are elevated as a result of loss of abomasal integrity (Simpson, 2000).

1.3 Immune response

1.3.1 Host immunity

In mammals, the immune system is divided into the innate and adaptive immune responses. The innate immune response is fast-acting, using non-specific lines of defence against invading pathogens and acts as a barrier to prevent infection. The adaptive response takes longer to develop, is highly specific and is termed the 'memory response'. The adaptive immune response has two further sub-divisions; the cellular-mediated and humoral response, of which the latter is involved with the production of antibodies specific to antigens from the invading pathogen. The ability to mount this

antibody driven response is maintained by B cells. If the host immune system encounters the pathogen for a second time, B cells are able to trigger the production of antigen-specific antibodies more rapidly; this is termed the anamnestic response.

$CD4^+$ T helper cells (Th) are a source of cytokines and have receptors which are able to recognise antigen peptides in association with major histocompatibility complex (MHC) presenting cells (Goldsby *et al*., 2003). Th cells can be further divided into subsets of cells which differ due to phenotype and function e.g. Th1, Th2 and Th17 (Goldsby *et al*., 2003). Th2 cells produce a number of cytokines including interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6) and interleukin-10 (IL-10) which drive a type 2 immune response and have been shown to have an important role in the control of helminth infections (Urban *et al*., 1995). These cytokine promote B cell proliferation, the secretion of immunoglobulins and mediate activation of mast cells and eosinophils (Goldsby *et al*., 2003).

The cellular-mediated response is associated with Th1 cells and is characterised by an activation and infiltration of effector cells to the site of infection. This cellular infiltration is in response to a specific antigen presented by a MHC molecule, and includes a cellular profile of macrophages and antigen-specific cytotoxic T-cells. This in turn can produce interferon-γ (INF- γ), interleukin-2 (IL-2) and interleukin-3 (IL-3) (Goldsby *et al*., 2003). These cytokines promote the production of activated macrophages, antibodies and mediate delayed type hypersensitivity responses and inflammatory reactions. This response is often elicited against invading intracellular organisms, for example viruses and bacteria.

1.3.2 Immunity against *T. circumcincta*

Parasitic gastroenteritis is primarily a disease of lambs as immunity is acquired after repeated exposure to the parasite (Halliday *et al*., 2007; Seaton *et al*., 1989). Repeated ingestion of larvae over a period of 8 – 12 weeks by sheep from the age of 5 months

results in a protective immune response (Abbott, Taylor and Stubbings, 2004). An experimental infection of 5 month-old lambs with *T. circumcincta*, showed that over an 8-week "trickle"-infection regime, with approximately 1,000 *T. circumcincta* L_3 administered weekly, animals were rendered immune to a subsequent challenge infection (Seaton *et al*., 1989). As acquired immunity develops, the lambs prevent incoming L_3 from establishing in the abomasum as well as provoking retardation in development of L_4 and influencing the growth, development and fecundity of adult worms (Balic, Bowles and Meeusen, 2000; Seaton *et al*., 1989; Stear *et al*., 2004).

1.3.3 Mechanisms of immunity

In immune sheep, an immediate type hypersensitive reaction, characterised by mast cell infiltration in the abomasal mucosa, is directed against incoming L_3. It is thought to be responsible, in part, for preventing the establishment of incoming larvae through expulsion or exclusion of larvae. This has been shown to occur as early as 2 days post-challenge (dpc) in sheep rendered immune by an experimental trickle infection of 10,000 *T. circumcincta* L_3 per week for a 9-week period and subsequently dosed with a bolus of 50,000 L_3 (Smith *et al*., 1984). It has been suggested that the overall burden is partly regulated by this immediate hypersensitivity response (Stear *et al.*, 1995). Continual exposure to nematode antigens, *i.e*. through grazing of contaminated pasture, induces and maintains the production of an antigen-specific IgA response in the abomasal mucosa (Smith *et al*., 1987). The relationship between developmental stage, IgA-reactive antigen profile and impact of *T. circumcincta*-specific abomasal IgA responses on abomasal worm burden has been explored using excretory/secretory (ES) products from *ex-vivo* exsheathed L_3 [day 1 post infection, (1dpi)], L_3/L_4 (3dpi) and L_4 (5dpi) and abomasal mucosal IgA from ewes rendered immune by trickle infection with 2,000 L_3 three times per week for 10 weeks (Smith *et al*., 2009). Immunoblots demonstrated L_3 and L_4 antigen-specific IgA in the abomasal mucus of immune ewes and an inverse relationship was observed between 3dpi ES-reactive abomasal IgA levels and worm

burden following challenge of sheep with 50,000 *T. circumcincta* L_3 (Smith *et al*., 2009). The abomasal mucosal IgA response has also been implicated in impairing growth and retarding L_4 development (Smith *et al*., 1985) and the proportion of larvae displaying arrested development is correlated with the magnitude of the local abomasal IgA response (Stear *et al*., 1995). In a study looking at the impact of immunosuppression on sheep infected with *T. circumcincta*, sheep were trickle-infected with 10,000 *T. circumcincta* L_3 weekly for a 6-week period and then subjected to a bolus challenge of 50,000 L_3 (Smith, 2007). In the trickle-infected animals, inhibition of L_4 development was triggered, but normal L_4 development was more rapidly resumed after administration of an immunosuppressive corticosteroid injection than in the non-immunosuppressed group (Smith, 2007), providing evidence that the proportion of arrested L_4 in the abomasum is controlled by the host immune response. In experimental infections with *T. circumcincta*, where yearling sheep were trickle-infected with 2,000 L_3 three times weekly for 2 months before challenge with 50,000 infective L_3, an abomasal mucus IgA anamnestic response to L_4 somatic antigen and L_4 ES was observed in efferent gastric lymph at 4-5 dpi of the bolus challenge and this response was linked to inhibited development of L_4 (Halliday *et al*., 2007). In addition to effects on development of L_4, the immune response to a persistent *T. circumcincta* challenge can impact the length of adult worms; for example, adult worms collected from the abomasa of sheep subjected to a trickle infection and bolus challenge of 50,000 L_3, were 1 mm shorter than those collected from sheep that were given a bolus challenge only (Halliday *et al*., 2007).

Antibody responses against gastrointestinal nematodes often are of greater magnitude and peak earlier after a secondary infection compared to primary infections. For example, in sheep infected with *Haemonchus contortus*, serum IgG responses to adult ES and somatic antigens increased within 24 hours and had higher titres following a secondary infection compared to a primary infection (Schallig *et al*., 1994; 1995). In experimental infections with *T. circumcincta*, yearling sheep were trickle-infected with 2,000 L_3 three times weekly for 2 months before challenge with 50,000 L_3, an

anamnestic response in both L_4 somatic antigen and L_4 ES-specific abomasal IgA levels was observed at 4-5 dpi and was linked to stunted L_4 growth (Halliday *et al*., 2007).

Immunity acquired under experimental conditions is linked to host age, with 10 month-old sheep having a greater proportion of inhibited L_4 than 4.5 month-old lambs, when infected under the same trickle infection regime. Here, both groups of lambs were trickle-infected with 2,000 L_3 five days a week for 9 weeks and then subjected to a 50,000 L_3 bolus challenge (Smith *et al*., 1985). A recent study contradicts this observation, as no significant difference was found between yearlings and 5 month-old lambs in nematode burden and/or the proportion of larvae in inhibited development under a similar trickle infection/bolus challenge protocol (Halliday, McAllister, and Smith, 2010). A possible explanation for these differences is that the 10 month-old sheep were less 'resilient' to the heavy trickle infection (2,000 L_3 five times per week for 8 weeks) used in the 1985 trial compared to the 2010 trial (2,000 L_3 three times per week for 8 weeks) (Halliday, McAllister, and Smith, 2010; Smith *et al*., 1985).

Protective immunity to *T. circumcincta* can be transferred directly between genetically identical sheep by the transfer of lymphocytes in gastric lymph collected from animals rendered immune by a trickle infection regime (Smith *et al*., 1986). 'Partial' protection was generated as evidenced by decreased worm burdens and stunted worm growth in recipients (Smith *et al*., 1986). In this experiment, stimulation of local IgA and mast cell infiltration in the mucosa implicated lymphocyte involvement in protective immunity.

T. circumcincta infection has also been associated with an increase in IgE levels (Huntley *et al.*, 1998a; 1998b). *T. circumcincta* L_3-specific IgE responses have been observed to peak between 2-8 dpc in sheep which had been administered a bolus challenge of 50,000 L_3 following a trickle infection (Huntley *et al*., 1998a; 1998b). These responses were shown to be directed towards antigens present on the cuticular surface of L_3. Importantly, the magnitude of the IgE response in lambs during their first grazing season, targeted towards *T. circumcincta* L_3 surface antigens, was associated

with low cumulative faecal egg counts (Huntley *et al*., 2001). A schematic overview detailing the current status of knowledge with regards to immunity to *T. circumcincta* is shown in Figure 1.3.

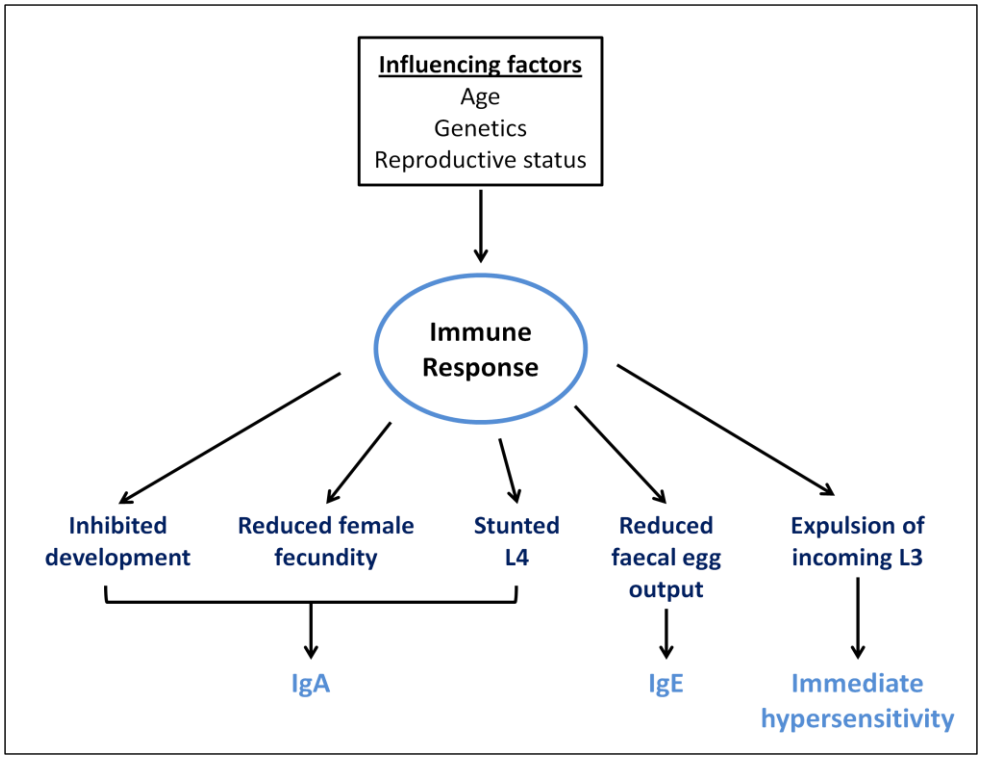

Figure 1.3 Summary of the known mechanisms and factors influencing the immune response to *T. circumcincta* infection in sheep.

1.3.4 Cellular immune response

During *T. circumcincta* infection, the cellular composition of the abomasal mucosa is characterised by infiltrates of T cells and eosinophils (Balic *et al*., 2003). The direct tissue damage caused by larval invasion and feeding has also been associated with eosinophil activation and pro-inflammatory cytokine release (De Veer, Kemp and Meeusen, 2007). Histopathological analysis of immune animals (but not primary infected, naïve animals) indicated that eosinophils are rapidly recruited and congregate with lymphocytes around invading larvae (McGillivery *et al*., 1992; Meeusen, Balic and Bowles, 2005). In previously infected sheep, following a 50,000 *T. circumcincta* L_3

challenge, mast cell infiltration and an increase in mucosal pepsinogen levels were linked to an immediate hypersensitivity response (Smith *et al*., 1984). Resistance to infection in previously infected animals has been associated with a secondary response. This has been indicated by a decrease in the proportion of $CD21^+$ cells in gastric lymph at 5 dpc (Balic et al., 2003; Smith et al., 1984). $CD21^+$ is a marker of immature B cells and its presence on the surface of B cells is lost when they fully mature into differentiated antibody secreting cells (Braun *et al*., 1998).

The kinetics measured in the cellular response and cytokine profile in immune animals highlight that *T. circumcincta* triggers a Th2 humoral response; characterised by up-regulation of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10) and interleukin-13 (IL-13) at 5 dpi (Craig *et al*., 2007; Miller, 1996). An increase in the up-regulation of cytokines associated with a Th1 cellular mediated response was also seen (Craig *et al*., 2007; Miller, 1996). This cytokine profile included interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-18 (IL-18), aswell as the transforming-growth factor-β (TGF-β), associated with tissue repair and collectively this indicates that there was a pro-inflammatory influence on the immune response generated following infection (Craig *et al*., 2007; Miller, 1996).

1.4 Control of parasitic gastroenteritis

1.4.1 Anthelmintics

Gastrointestinal nematode infections in small ruminants are controlled by the application of grazing management strategies combined with the use of broad-spectrum anthelmintics. Anthelmintic treatment can be used strategically to prevent disease in lambs which have not yet acquired immunity to the parasites (Abbott, Taylor and Stubbings, 2004; Prichard *et al*., 2007). There are five classes of anthelmintics licensed in the UK for administration to small ruminants: benzimidazoles, imidothiazoles, macrocyclic lactones, amino-acetonitrile derivatives (AADs) and spiroindole

compounds. In the early 1960s, the first benzimidazole, thiabendazole, was introduced. The mode of action is linked to binding to β-tubulin, preventing microtubule formation, which is critical to maintain the integrity and function of parasite cells (Pritchard, 1990). Due to the disruption in cellular processes, cell lysis negatively impacts upon parasites motility and feeding (McKellar and Jackson, 2004; Mitreva *et al.*, 2007). The imidothiazole class of anthelmintics was first developed in the 1970s; these are nicotinic agonists causing depolarisation of neuromuscular junctions and paralysis of the parasite musculature, resulting in expulsion of the parasite from the host (Martin *et al.*, 1998; McKellar and Jackson, 2004). Licensing of the macrocyclic lactones in 1981 represented an important tool in control, as these were the first anthelmintic class to have efficacy against both ectoparasites and endoparasites and had a level of persistence in their activity (Geary, 2005). The macrocyclic lactones class is split into two groups; avermectins (for example ivermectin) and milbemycins (for example moxidectin). The macrocyclic lactones are thought to exert their mode of action by irreversibly binding to gamma-aminobutyric acid- (GABA) and glutamate-gated chloride (GluCl) channels resulting in paralysis of pharyngeal and somatic muscle cells, which in turn causes immobility of the worms (Blackhall *et al.*, 1998; Feng *et al.*, 2002; Martin *et al.*, 1998; Wolstenholme *et al.*, 2004). One of the most recent classes of anthelmintic to be introduced is AADs, which have as a target nicotinic acetylcholine receptors (Kaminsky *et al.*, 2008; Prichard and Geary, 2008). Like other broad-spectrum anthelmintics, their mode of action is to induce paralysis of somatic muscle cells and they are efficacious against larval and adult stages of gastrointestinal nematodes (Besier, 2009; Kaminsky *et al.*, 2008). The latest class of anthelmintic are the spiroindoles, which are semi-synthetic molecules and their mode of action is to block cation ion channels in somatic muscle cells (Little *et al.*, 2011), ultimately inducing paralysis of the nematode.

1.4.2 Anthelmintic resistance

The selection pressure of frequent anthelmintic treatments that has been exerted on parasitic nematode populations has lead to the development of resistance to some of the

broad spectrum classes. Anthelmintic resistance is irreversible and is heritable between generations of worms (Jackson and Coop, 2000; Wolstenholme *et al*., 2004). *Teladorsagia* is now the predominant resistant genus in the UK (Bartley *et al*, 2003; Kaplan, 2004) and isolates of *T. circumcincta* which are resistant to all three classes of commonly used anthelmintics (benzimidazoles, imidothiazoles and macrocyclic lactones) have been reported (Bartley *et al*, 2004; Sargison *et al*, 2007). The presence of multi-drug resistance on a farm can result in the suspension of sheep farming on the property until the parasite contamination on grazing pasture is removed. It is difficult to ascertain the full extent of anthelmintic resistance in the UK as no routine surveillance surveys for resistance are conducted and it is often diagnosed after clinical signs of drug failure on a farm have been reported (Sargison *et al*., 2001; 2007). Anthelmintic resistance is a major threat to livestock production worldwide; however it has a higher prevalence in the Southern Hemisphere (Jackson and Coop, 2000; Kaplan, 2004). As the AADs have recently been licensed there are no reports of field resistance to this class yet; however, *in vitro* experiments were able to select AAD-resistant *Caenorhabditis elegans* and molecular resistance markers were identified (Kaminsky *et al*., 2008).

1.5 Management strategies

There are currently no effective alternatives to anthelmintics for the control of gastrointestinal nematodes in sheep, so other strategies are required. A number of alternative control methods have been investigated, including targeted selective treatments, pasture management, nutritional supplementation, selective breeding and vaccination (Athanasiadou, Houdijk and Kyriazakis, 2008; Coop and Kyriazakis, 2001; Greer *et al*., 2007; Hoste *et al*., 2002; Hunt, McEwan and Miller, 2008). Grazing management strategies are used as a means to reduce the selective pressure on parasites, to slow development of resistance mechanisms, by reducing the levels of pasture contamination. A proportion of the parasite population will not be exposed to anthelmintics. These parasites, often on pasture, are termed '*in refugia*'. It is thought

that if a proportion of the flock were not subjected to anthelmintic treatment, the number of worms *in refugia* would be increased. This strategy has been adapted for the control of *H. contortus*, in which small ruminants are prioritised for anthelmintic treatment by comparing the colour of the conjunctiva mucus membrane to a colour chart to assess the level of anaemia (Van Wyk and Bath, 2002). Animals with no clinical signs of parasitic infection are left untreated and add to the proportion of *in refugia* worms on pasture. This selective system has been developed further in terms of targeting treatment towards factors influencing production and animal performance, including live weight gain, body condition and faecal worm egg counts (FWEC), and has shown considerable promise as an effective management strategy (Besier, 2008; Greer *et al*., 2009; Kenyon *et al*., 2009; Stafford *et al*., 2009). Other grazing management strategies include co-grazing of sheep and cattle, age-grouped grazing and rotation of pasture (Abbott, Taylor and Stubbings, 2004; Waller and Thamsborg, 2004).

Selective breeding strategies have been used to 'select' for the individual sheep in a flock which have a greater resilience to parasite infection, *i.e.* they are able to reach production targets whilst infected (Stear *et al*., 2000). Using genetic selection, it may be feasible to breed a line of animals that have greater resilience to infection and/or a better level of immunity. The choice of measure used for genetic selection needs to be further defined, as selecting for individuals with low cumulative FWECs in isolation from other measures of production may be 'hiding' those sheep under high physiological pressure to maintain their level of resilience (Greer *et al*., 2008).

1.6 Vaccine development

Vaccination against gastrointestinal nematodes would be a desirable alternative control strategy to anthelmintic treatments. To date, there have been few commercially available helminth parasite vaccines produced. The only one available for use in ruminants is Bovilis® Huskvac (MSD Animal Health), which was developed for the

control of lungworm in cattle and is based on oral administration of live attenuated L_3 (Jarrett *et al.*, 1955; Vercruysse *et al.*, 2004).

Vaccination of sheep against *T. circumcincta* is a possible option as a protective response can be acquired in the host following repeated exposure to the parasite either through natural field exposure or experimental infection (Smith *et al.*, 1983; Stear *et al.*, 1999). A vaccination programme could potentially be used in conjunction with existing strategies to prolong anthelmintic effectiveness, reduce pasture contamination and to protect the most susceptible sub-group of animals within a flock (Emery, McClure and Wagland, 1993). Successful vaccination could allow lambs to develop immunity before factors such as stress, inadequate nutrition and pasture contamination puts them at high risk of infection (McClure, 2009). The development of a vaccine against parasitic nematodes requires a detailed knowledge of the biology of the parasite, the nature of interactions with the host and the effective immune responses that are generated during infection (Knox, 2010). The first published set of *T. circumcincta* vaccination experiments, in the late 1970's, used a mixture of L_3 and L_4 ES products from *ex-vivo* worms as antigen and induced 69% reduction in worm burden in vaccinated animals compared to identically-challenged adjuvant-only, recipients (Rose, 1976; 1978).

1.6.1 Approaches used in nematode vaccine development

Development of vaccines against parasitic nematodes has involved targeting molecules from different stages of the parasite life cycle, including products excreted/secreted from larvae or exposed on the parasite surface, using local antibody probes (Murphy *et al.*, 2010; Nisbet *et al.*, 2010a; Redmond *et al.*, 2006; Smith *et al.*, 2009) or the identification of molecules with homology to other known promising vaccine candidates (Nisbet *et al.*, 2009; 2010b; 2011). A successful anti-nematode vaccine is likely to be one that will target several antigens expressed by various developmental stages of the parasite (Knox, 2010).

1.6.1.1 L_3 antigens

The L_3 represents the first stage to come into contact with the host and its immune system, and therefore is critical in the life cycle as it could determine if the parasite is established within the host or excluded from the gastrointestinal system. One of the first immunogenic antigens to be identified from *T. circumcincta* L_3 was a 31 kDa molecule located to the secretory organelles (McGillivery *et al*., 1989; 1992). This molecule displayed promise as a vaccine candidate in an immunisation trial where the 31 kDa antigen was purified from a detergent extract of *T. circumcincta* L_3, 400µg of the antigen administered with Quil A adjuvant to 6 month-old sheep and, following immunisation, the sheep subjected to a single bolus challenge of 42,000 *T. circumcincta* L_3 and antibody responses and parasitological parameters monitored (McGillivery *et al*., 1992). Comparison of vaccinated sheep to adjuvant-only controls showed reductions in both FWEC and worm burdens (42%) alongside the presence of antigen-specific serum IgG as early as 4 days after administration of the first vaccine dose (McGillivery *et al*., 1992). However, attempts to replicate this result have been unsuccessful as cell-mediated and antibody responses were not induced (Morton *et al*., 1995). One of the reasons behind this is that challenge dose given was a non-natural large dose and the immunity afforded by the vaccination may not have been adequate to control the parasite infection. Another potential vaccine candidate from *T. circumcincta* L_3 was identified through the bioinformatic analysis of a stage-specific expressed sequence tag (EST) dataset from exsheathed *T. circumcincta* L_3 (Nisbet *et al*., 2008; 2009). This surface associated antigen, Tci-SAA-1, shared over 77% amino acid identity with Ac-SAA-1, a vaccine candidate from the canine hookworm, *Ancylostoma caninum*, and was localised to the sub-hypodermal layer below the cuticle of exsheathed *T. circumcincta* L_3 (Nisbet *et al*., 2009). Abomasal mucus IgA obtained from 5 month-old lambs which had been subjected to an experimental trickle infection (2,000 *T. circumcincta* L_3 three times per week for a 10-week period) bound to the bacterial-expressed recombinant version of Tci-SAA-1, and anti-Tci-SAA-1 antibodies recognised a 12-14 kDa antigen present only in somatic and surface extracts of L_3 (Nisbet *et al*., 2009). Tci-SAA-1 is therefore a

promising vaccine candidate, primarily due to the high level of homology with *A. caninum* vaccine candidate, localisation on the surface of exsheathed *T. circumcincta* L_3 and the potential of the antigen to be a target for abomasal mucus IgA. Surface antigens therefore possess promise as vaccine candidates; however the external surface of parasitic nematodes is complex and dynamic. The outer covering of the nematode, termed the cuticle, is a multilayered structure with a unique structural and chemical composition (Blaxter *et al*., 1992). A carbohydrate-rich surface coat, termed the 'glycocalyx' is associated with the epicuticle and has been implicated in immune evasion (Maizels *et al*., 1993). In addition, following ingestion of infective *T. cirucmcincta* L_3, the L_3 exsheath, develop and moult, and the profile of the surface antigens displayed alters (Keith *et al*., 1990).

Surface antigens of *T. circumcincta* L_3, have been stripped from the epicuticle using detergent and subsequently used in an immunisation trial to investigate their ability to induce a protective response in sheep against challenge (Wedrychowicz *et al*., 1992; 1995). Sheep vaccinated with L_3 surface extracts in conjunction with beryllium hydroxide adjuvant and then challenged with a single bolus of 50,000 L_3 had a 72% reduction in worm burden compared to adjuvant-only recipients (Wedrychowicz *et al*., 1992; 1995). Vaccination with L_3 surface extracts induced a humoral response, with vaccinated sheep having a higher level of L_3 surface antigen-specific serum IgA and IgG compared to control sheep (Wedrychowicz *et al*., 1992). However, when repeated in 1995, the same level of protection was not seen as there were no differences in serum IgA responses between vaccinated and control sheep (Wedrychowicz *et al*., 1995).

An L_3-specific carbohydrate-larval antigen (CarLA), was found to be present on the cuticular surface of numerous nematode species, including *Trichostrongylus colubriformis, T. circumcincta, H. contortus, Nematodirus americanus* and *Cooperia curticei* (Harrison *et al*., 2003a; 2003b). Studies with *T. colubriformis* found that antibodies specific to CarLA were strongly associated with immune-mediated rejection of incoming L_3, suggesting that CarLA is implicated in L_3 establishment in the tissue niche (Harrison *et al*., 2003a; 2008). However, further research has revealed that there

is variation in the versions of CarLA antigens displayed on individual worms, *i.e.* individual larvae have different versions of CarLA (Maas *et al*., 2009). The implication of CarLA in L_3 establishment and the finding that anti-CarLA antibodies mediate rejection of incoming L_3 into the abomasum highlight that carbohydrates displayed on the surface of nematode parasites have the potential to be recognised by the host immune system.

1.6.1.2 L_4 antigens

At the L_4 stage, the parasite has already established in the abomasum and a vaccine that targets this stage could inhibit or prevent mechanisms by which larvae survive and grow. In the abomasal gland, L_4 parasites release ES products and these could be a critical area to target to interrupt interactions between the host and parasite. Nematode ES products have been identified as important sources of protective antigens in other ruminant species (Meyvis *et al*., 2007). In an attempt to identify some of the components of *T. circumcincta* L_4 ES products, and hence possible vaccine candidates, a proteomic analysis of L_4 ES products collected at 8 dpi was conducted (Redmond *et al*., 2006). Analysis revealed that the most abundant protein present in L_4 ES products was the proteolytic enzyme cathepsin-F (Tci-CF-1) (Redmond *et al*., 2006). This was a proposed vaccine candidate due to other members of the cathepsin family being validated vaccination targets in other helminth species (Dalton *et al*., 2003; Tort *et al*., 1999) and because of the essential roles proteinases play in nematode survival and development, including moulting, tissue invasion, digestion and evasion of host immune responses. Tci-CF-1 was a proven target of the local immune response in sheep subjected to a trickle infection and bolus challenge regime, as abomasal mucus IgA levels specific to Tci-CF-1 corresponded to the observed peak in total IgA in efferent gastric lymph (Redmond *et al*., 2006). A later study also used a proteomics approach to identify key molecules in *T. circumcincta* L_3 and L_4 ES products (Smith *et al*., 2009) from worms collected at 1, 3 and 5 dpi. Tci-CF-1 was identified in L_4 ES products

collected from worms harvested at 3 dpi (Smith *et al*., 2009). Another potential vaccine candidate identified by the proteomic analysis of L_4 ES products was an activation-associated-secreted protein (ASP), Tci-ASP-1 (Nisbet *et al*., 2010; Smith *et al*., 2009). ASPs are nematode-specific members of a diverse protein family, called SCP/Tpx-1/Ag5/PR-1/Sct (SCP/TAPS), present in eukaryotes and a range of parasites (Cantacessi *et al*., 2009; 2012). The first report of their existence in parasites was in the ES products of serum-activated L_3 of the canine hookworm, *A. caninum* (Hawdon *et al*., 1996; 1999). They are key vaccine candidates against hookworm species and are thought to be important in the establishment of parasites in the host (Hawdon *et al*., 1999; Tawe *et al*., 2000). Tci-ASP-1 was found to be specific to the parasitic stages of *T. circumcincta,* and a bacterial-expressed recombinant version was shown to be the target of abomasal mucus IgA obtained from sheep rendered immune to challenge through a prolonged experimental trickle infection/challenge regime (Nisbet *et al*., 2010).

1.7 Current challenges in parasitic nematode vaccine development

A number of studies have reported difficulties in stimulating the same levels of protective immunity with recombinant vaccine candidate antigens as had been observed when native versions of parasite molecules or extracts were tested (Meyvis *et al*., 2008; Smith and Zarlenga, 2006). Abomasal mucus IgA has been a useful immunological probe to identify a number of possible protective antigens from *T. circumcincta* which have displayed promise as potential vaccine candidates, including Tci-CF-1, Tci-ASP-1 and Tci-SAA-1 (Nisbet *et al*., 2009; 2010a; 2010b; Redmond *et al*., 2006; Smith *et al*., 2009). These molecules, as well as five others identified as immunogenic or immunostimulatory during the sheep/*T. circumcincta* interaction, have recently been formulated as a recombinant cocktail which, when administered to 5-6 month-old sheep, induced 58-70% reductions in cumulative FWEC during a *T. circumcincta* trickle challenge (Nisbet *et al*., 2013). Despite this success, the complexity of the vaccine

formulation may preclude its appeal for commercial exploitation. For other species of parasitic nematode there has been limited success in the development of recombinant subunit vaccines. There are several potential reasons likely to underlie these failures. Perhaps the most important are unsuitable expression systems for antigen production leading to incomplete or inadequate post-translational modifications; for example, glycosylation and tertiary protein folding, resulting in incorrect epitope structures for antibody binding (Geldhof *et al*., 2007).

1.7.1 Glycosylation

Glycosylation of nematode proteins can enhance the immunogenicity of antigens or be involved in the modulation of the host's immune response (Dell *et al*., 1999). In some cases, carbohydrate components of antigens have been implicated as having a potential role in antibody recognition; for example, novel glycan epitopes were identified in adult *H. contortus* ES products and the glycan specific antibody titres correlated with protection (Van Stijn *et al*., 2010; Vervelde *et al*., 2003). In particular, antibody recognition of two glycan epitopes, Galα1-3GalNAc-R and GalNAcβ1-4(Fucα1-3)GlcNAc-R (LDNF), was found to be significantly correlated with a protective serum IgG response against *H. contortus* in sheep immunised with adult worm ES products (Van Stijn *et al*., 2010; Vervelde *et al*., 2003). Furthermore, binding of ES-antigen-specific serum IgA and IgG decreased if ES antigens had been treated with sodium periodate prior to analysis by ELISA (Vervelde *et al*., 2003), indicating that the structure of the glycans were involved in antibody binding. From this, Vervelde *et al*. (2003) concluded that ES glycan components in *H. contortus* were immunogenic, suggesting that the glycan-specific immune response may contribute to protection against nematode infection. Research into a lead vaccine candidate for *H. contortus*, termed *Haemonchus* galactose containing glycoprotein complex (H-gal-GP) (Smith *et al*., 2000), identified the presence of the LDNF glycan on a metalloendopeptidase (MEP-3) component of H-gal-GP (Geldhof *et al*., 2005). In contrast to the work presented by Vervelde *et al*., (2003), there was no relationship found between the antibody response to the LDNF

glycan on H-gal-GP and the level of vaccine induced protection in lambs (Geldhof *et al*., 2005). In more detail, there was no significant correlation between the level of antibodies specific to the LDNF glycan found on H-gal-GP and the level of protection afforded in sheep immunised with native-H-gal-GP or denatured, reduced H-gal-GP following a challenge infection of 5000 *H. contortus* L_3 (Geldhof *et al*., 2005).

Carbohydrate residues have been found on components of ES products and somatic extracts from a range of parasitic nematodes including *Dictyocaulus viviparus* (Haslam *et al*., 2000), *H. contortus* (Haslam *et al*., 1996; Vervelde *et al*., 2003) and *Toxocara canis* (Maizels and Page, 1990). N-linked glycans, in particular a glycan epitope termed 'Lewis^x' (Galβ1-4(Fucα1-3) GlcNAc), were present in detergent extracts of *D. viviparus* (Haslam *et al*., 2000). Lewis^x type projections on bi-, tri- and tetra-antennary molecules were identified in an extract from adult *D. viviparus*, which potentially have an immunomodulatory role (Haslam *et al*., 2000). Phosphorylcholine modifications of N-linked glycans represent another proposed immunomodulatory glycan structure and these have recently been identified in the porcine nematode *Ascaris suum* from an antigen preparation of adult worms (Poltl *et al*., 2007). In general, parasite glycans are often more structurally complex than mammalian glycans; this is primarily related to parasite-specific modifications to the core glycan structures (Hein and Harrison, 2005; Nyame, Kawar and Cumming, 2004). Consequently, this means that recombinant expression systems may not be able to manufacture the glycoprotein with the relevant specific modifications, for example, terminal sugar composition, elongation of chain length and variation in the composition of linkage patterns, that are required for the range of epitopes that are able to stimulate specific antibodies when the native molecule is presented to the host immune system (Khoo and Dell, 2001). When glycans prepared from the free-living nematode *C. elegans* were used as immunogens in an immunisation trial against *H. contortus* in sheep, no protection against challenge with the parasitic nematode was observed, despite extensive homology between the glycans that have been identified in both nematode species (Cipollo, Costello and Hirschbeg, 2002; Haslam, Coles and Munn, 1996; Redmond, Geldhof and Knox, 2004).

Similarities are evident in the two nematodes' glycosylation patterns; both having high mannose content and complex N-linked glycans (Cipollo, Costello and Hirschbeg, 2002; Haslam, Coles and Munn, 1996). *C. elegans* has also been used as a heterologous protein expression system for *H. contortus* antigens, to attempt to introduce appropriate glycosylation and protein folding and when Hc-CPL-1 (cathepsin L cysteine protease of *H. contortus*) was expressed in *C. elegans*, the resultant recombinant protein was enzymically active and glycosylated. However, intramuscular immunisation with this antigen failed to protect sheep against challenge with *H. contortus* (Murray *et al*., 2007). These studies reflect the complexity of both candidate antigen selection and heterologous expression systems in the generation of host-protective nematode vaccines.

1.7.2 Conformational epitopes

There are two classifications of epitopes on protein antigens, linear and conformational. These are based on the structure of the epitope and its antibody interaction (Huang and Honda, 2006). A linear epitope is formed by amino acid residues that are present on the antigen in a linear sequence (Figure 1.4). A conformational epitope, also known as structural or discontinuous, is formed when the amino acid residues are on distinct, separate areas of the protein antigen. Upon tertiary protein folding, the residues are brought into close proximity and form the three-dimensional epitope required for specific antibody recognition (Figure 1.4).

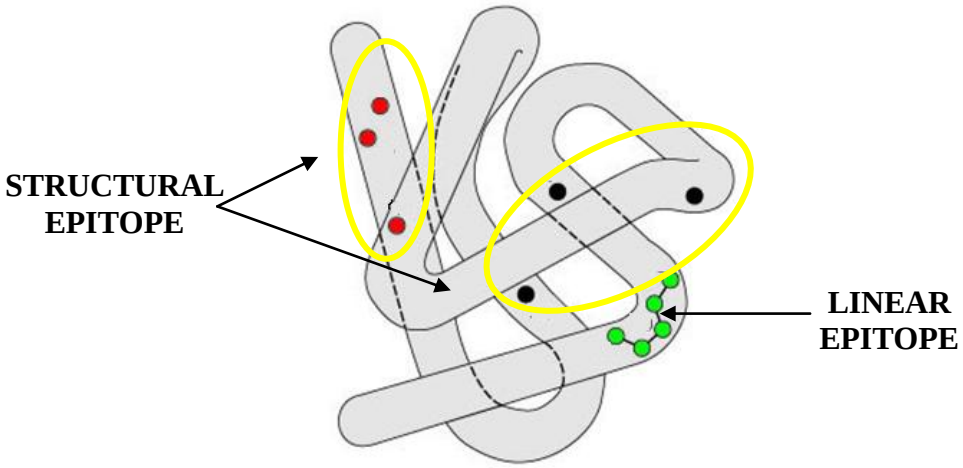

Figure 1.4 Schematic illustration of linear and conformational epitopes.

(Image adapted from Roitt's Essential Immunology, 11th ed). Green dots represent linear peptides connected to create a linear epitope for antibody recognition. Red and black dots (highlighted by yellow ovals) are on distinct areas of the antigen. Following tertiary protein folding they are brought into close proximity and represent a discontinuous, structural/conformational epitope.

The relative importance of correct glycosylation in stimulating appropriate immune responses may be difficult to isolate from the impact of other post-translational modifications, such as protein folding, tertiary and quaternary structures and the formation of protein complexes (either homologous or heterogenous). This is illustrated by studies on immunisation of cattle against the abomasal nematode, *O. ostertagi*, using activation-associated secreted proteins (ASPs) (Geldhof *et al*., 2008). The proteins in question, Oo-ASP-1 and Oo-ASP-2, were the principal components of an enriched native ES preparation derived from adult *O. ostertagi*, which had been shown previously to successfully immunise cattle against challenge (Geldhof *et al*., 2002; 2004; Meyvis *et al*., 2007). However, vaccination with a recombinant version of Oo-ASP-1, produced in a eukaryotic (baculovirus/insect cell-based) expression system, failed to induce protective immunity or native-antigen-specific serum antibodies in immunised calves (Geldhof *et al*., 2008). In these studies, it was initially suggested that incorrect or inadequate glycosylation may be responsible for the absence of protective immunity or the lack of native antigen-specific antibodies after vaccination. However, deglycosylation of native Oo-ASP-1 and Oo-ASP-2 antigens using peptide-N-glycosidase F (PNGase F) had no deleterious effect on the binding of serum antibodies

(IgG_1, IgG_2, IgM or IgA) from native antigen-immunised calves to these proteins (Meyvis *et al*., 2008). Further investigations demonstrated that denaturation of the native ASPs using the reducing agent dithiothreitol reduced their ability to be bound by antigen-specific antibodies, suggesting that these antibodies are directed against epitopes formed by secondary or tertiary structure arrangements rather than primary protein sequence or glycan. This study (Meyvis *et al*., 2008) highlighted that the ability of discontinuous peptide sequences to form conformational epitopes can be essential for retaining antibody recognition, and confirms that tertiary or quaternary protein folding are critical post-translational modifications for reproducing responses manifested by nematode antigens in native form.

1.7.2.1 Protein folding

Further evidence to support the importance of the correct protein folding, and thus structural epitope formation, can be shown by investigations into the potential role of N-linked glycans in immunologically-mediated protection against *D. viviparus.* Several antigens from an L_3 extract and adult ES products contained N-glycan residues (Kooyman *et al*., 2007). Following deglycosylation of these antigens with PNGase F treatment, the degree of antibody binding by serum antibodies from vaccinated cattle was reduced indicating that the N-glycan residues were important for antibody recognition (Kooyman *et al*., 2007). Further investigation of the role of N-glycans in the protective immune response used a comparison of serum IgG_1 titres specific to L_3 and adult ES antigens between re-infected calves (primary infection followed by challenge) and a primary infection (challenge control) group. Following challenge, the antigen-specific antibody titres increased for both groups (Kooyman *et al*., 2007). On deglycosylation of antigens, the level of antibody recognition was significantly higher in the re-infected group compared to the primary infected calves, highlighting that, although N-glycans were responsible for the induction of a strong immune response, it was in fact the protein backbone that was implicated in generation of the anamnestic response (Kooyman *et al*., 2007). Collectively, this suggests that it is essential that a

recombinant protein antigen is folded correctly in order to present the necessary epitopes for antibody binding.

The role of conformational or structural epitopes in immunity against nematodes has been a focus during vaccine development against *H. contortus* (Munn *et al.*, 1997; Smith and Smith 1996; Smith *et al.,* 2000). In a vaccine trial with the glycoprotein antigen, H11, a microsomal aminopeptidase isolated from the interstinal microvilli of *H. contortus* (Smith *et al*., 1994), the antigen was denatured using sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) (Munn *et al*., 1997), to disrupt the epitope structure of the native antigen preparation, and compared with non-denatured (native) H11. Following vaccination of sheep and challenge infection with 10,000 *H. contortus* L_3, FWECs and worm burdens were 21% and 41% higher, respectively, when the H11 antigen was denatured compared to the untreated native antigen (Munn *et al*., 1997). This demonstrates that structural epitopes on the native antigen were important for antibody binding and hence facilitated the protective response induced by vaccination with the native antigen.

Further evidence for the importance of structural epitopes is provided by research into a lead vaccine candidate for *H. contortus*, termed *Haemonchus* galactose-containing glycoprotein complex (H-gal-GP) (Smith *et al*., 2000), which was identified and fractionated from the brush border of adult worm gut membranes by lectin affinity chromatography (Smith *et al*., 1994). Vaccination with native H-gal-GP antigens induced 90% and 70% reductions in FWECs and adult nematode burdens, respectively, following challenge infection of yearling sheep with *H. contortus* L_3 compared to unvaccinated sheep (Smith *et al*., 1994; 2000). To identify components of the complex, native H-gal-GP was dissociated and fractionated by gel filtration chromatography, revealing a high molecular weight fraction that contained four metalloendopeptidases (MEPs) (Smith *et al*., 2003a) and a low molecular weight fraction that contained pepsin-like aspartyl proteases (Smith *et al*., 2003b). When these antigen fractions were tested as vaccine candidates, both the MEPs and the aspartyl proteases reduced faecal egg output by around 50% when compared with challenge controls (Smith *et al* 2003a;

2003b). When the MEPs were further fractionated by non-reducing SDS-PAGE and electro-elution, they performed less well than the urea-dissociated MEPs, in that vaccination resulted in only a 33% reduction in faecal egg output and 10% reduction in worm burden after L_3 challenge (Smith *et al*., 2003a). When the antibody responses from each group were tested by ELISA against non-denatured native H-gal-GP antigen, the urea-dissociated and SDS-PAGE-electroeluted MEP groups had antibody titres that were at least as high as the group vaccinated with non-denatured antigen (Smith *et al*., 2003a). However, it is clear from these experiments that even high titre antibody directed against primary antigen structure (the antibody produced in response to fully reduced and denatured antigen) was not sufficient to produce a protective effect against challenge, whereas antibodies raised specifically against non-denatured H-gal-GP antigen were highly protective. This further highlights the importance of structural antigens in antibody binding. These studies on H-gal-GP indicate that structural epitopes on an antigen complex are essential for antibody binding - the immunity afforded by vaccination with the complex H-gal-GP is directed against the 'whole' complex, as vaccination with individual components failed to reproduce the same level of protection afforded with the whole complex (Smith *et al*., 2003a; 2003b).

As a result, it is necessary to investigate conformational epitopes to design more effective anti-nematode vaccines, and one method, that has shown potential, is to exploit the idea that peptide sequences can 'mimic' the conformation of an epitope. These peptides can be identified via various applications of phage-display technology by panning with host-protective antibodies. Phage-display libraries of random peptides are now commercially available, and phage clones of interest are identified by a technique known as 'biopanning'. Currently, the most promising application of biopanning phage-display libraries is for the identification of conformational epitopes. This is achieved through biopanning phage libraries using purified antibodies as the immobilized target to identify those epitopes that are specifically identified by the antibody. These epitopes will include some that represent primary amino acid sequence and, more frequently,

those that can be considered as a 'peptidomimetic' of conformational epitopes (Smith and Scott, 1993).

In summary, further knowledge and exploitation of the antigenic epitope structure(s) of candidate nematode vaccine antigens are essential for the development of a successful anti-nematode vaccine. The structural composition of conformational epitopes, whether involving glycans, discrete peptide composition or discontinuous sections of amino acids which, following folding and tertiary structure formation, come into close proximity, has been demonstrated to be essential in achieving protection against parasitic nematodes in a number of models. As a result, it is necessary to investigate conformational epitopes through using phage-display libraries to design more effective anti-nematode vaccines.

1.8 Project aims and objectives

As a novel approach in the development of a vaccine against *T. circumcincta*, the aim of this project is to employ phage display libraries to identify peptide sequences that mimic the structure of native antigenic epitopes in somatic and surface extract preparations of *T. circumcincta* L_3. Thus, the objectives of the project are as follows:

1. The immunoreactivity of antigens present in extracts of the parasitic stages of *T. circumcincta* will be investigated through immunological techniques using local antibody probes generated from sheep which had been previously exposed to infection. Furthermore, the role of carbohydrate moieties in the generation of local immune responses against the parasite will be investigated.
2. An immunoaffinity chromatography approach will be used to purify native IgA-reactive antigens from somatic extracts of *T. circumcincta* L_3. A proteomic analysis of the purified IgA-reactive fraction will be undertaken to identify these proteins. The relative immunogenicity of the selected antigens will then be assessed by measuring gastric lymph IgA responses in experimentally infected

sheep demonstrated by parasite burden analysis to have varying degrees of immunity.

3. Phage display libraries will be used in an attempt to identify peptide sequences that mimic structural epitopes of *T. circumcincta* L_3 antigens. Abomasal mucus from sheep which had previously been infected with the parasite will be used to enrich two antibody pools for biopanning the libraries:
    1) IgA antibodies that bound *T. circumcincta* L_3 somatic antigens;
    2) Antibodies that bound native antigens on the surface of live, intact exsheathed *T. circumcincta* L_3.
4. The relative immunogenicity of both the native IgA-reactive antigens and selected peptide sequences from the two pools of phage clones will then be assessed by examining local immune responses to them in sheep with varying levels of acquired immunity to *T. circumcincta* infection.

Chapter 2 : Immunoreactivity of *Teladorsagia circumcincta* larval antigens

2.1 Introduction

Gastrointestinal nematode parasites are a major constraint to the sheep farming industry worldwide due to economic and welfare issues. In temperate regions of the world, one of the most prevalent gastrointestinal parasites is *Teladorsagia circumcincta* (Bartley *et al*., 2003). This nematode is currently controlled using a combination of anthelmintic treatments and pasture management; however, resistance is widespread and multi-class resistance is being reported increasingly (Bartley *et al*., 2004; Sargison *et al*., 2007). With the emergence of anthelmintic resistant parasites, this has created an interest in developing vaccines as an alternative means of control. Vaccination is a possible option because protective immunity can be induced after repeated exposure to the parasite through natural exposure or experimental infection (Smith *et al*., 1983; Stear *et al*., 1999). A number of studies have therefore focused on the immunological basis of host resistance to *T. circumcincta* infection. The majority of these have been experimental studies, which have been designed to mimic the challenge on pasture (Beraldi *et al*., 2008). From these, it is evident that a protective immune response can control *T. circumcincta* infection at several stages of its development (Halliday *et al*., 2007; Smith *et al*., 1985). In recent years, a number of promising vaccine candidates have been identified, including cathepsin-F (Tci-CF-1) (Redmond *et al*., 2006), activation-associated secreted protein-1 (Tci-ASP-1) (Nisbet *et al*., 2010a; Smith *et al*., 2009) and L_3-surface-associated antigen (Tci-SAA-1) (Nisbet *et al*., 2009). Vaccine development strategies have involved either targeting molecules from different stages of the parasite's life cycle, including the targeting of products excreted/secreted from worms or exposed on the parasite's surface, with local antibody probes or the identification of molecules with homology to other known promising vaccine candidates.

A ‘hypersensitivity reaction’ characterised by mast cell infiltration in the abomasal mucosa is directed against incoming L_3 and is thought to be responsible for exclusion and/or expulsion of larvae from as early as 2 days following challenge in previously infected sheep (Seaton *et al*., 1989; Smith *et al*., 1984). In an initial study by Seaton *et al*. (1989), 5 month-old lambs were administered 1,000 L_3 per week for a period of 4–12 weeks, followed by a challenge dose of 3,000 L_3 and the results indicated that, as the duration of the exposure period increased, the number of worms recovered at post mortem decreased. Evidence of an immune response targeted towards incoming L_3 has also been indicated during a trickle infection of 2,000 L_3 per day, 5 days a week for a total of 9 weeks, before administration of a 50,000 L_3 challenge (Smith *et al*., 1984). In this experiment, the 10 month-old trickle-infected sheep were necropsied at different time-points after challenge, and there was evidence of a rapid expulsion of incoming L_3 from as early as 2 dpc (Smith *et al*., 1984). An effective immune response targeted towards incoming L_3 would therefore potentially affect the proportion of larvae that establish in the abomasum (Balic *et al*., 2003). The local IgA response in the abomasum has been proposed as an important component of protection; negative correlations have been found between L_4 length and lymph IgA concentrations in experimental infections of 5 month-old lambs (Smith *et al*., 1985) and after natural exposure on pasture in 6-7 month-old lambs (Strain *et al*., 2002). Due to its role in protective immunity against *T. circumcincta*, abomasal mucus IgA has been used to identify a number of possible protective antigens (Redmond *et al*., 2006).

Despite this work, there has been limited success in developing recombinant subunit vaccines against gastrointestinal nematodes of livestock (Ellis *et al*., 2012). There are many potential reasons likely to underlie these apparent failures. Perhaps the most important are unsuitable expression systems for antigen production leading to incomplete or inadequate post-translational modifications; for example, glycosylation and tertiary protein folding, resulting in incorrect epitope structures for antibody binding (reviewed in Geldhof *et al*., 2007). Glycosylation of nematode proteins can enhance the immunogenicity of antigens or be involved in modulation of the hosts’ immune response

(Dell *et al.*, 1999) and the glycocalyx, which is a surface coat overlying the nematode cuticular surface, is abundant in carbohydrate (Blaxter *et al*., 1992). Research has shown that nematode glycans have been found in immunogenic antigens of parasitic helminths; for example, *Dictyocaulus viviparus* (Kooyman *et al*., 2007) and *Haemonchus contortus* (Schallig and Leeuwen, 1996). In particular, two glycan epitopes, Galα1-3GalNAc-R and GalNAcβ1-4(Fucα1-3)GlcNAc-R, were proposed to be implicated in the induction of a protective serum IgG response against *H. contortus* in sheep immunised with adult worm ES products (Van Stijn *et al*., 2010; Vervelde *et al*., 2003). Carbohydrate residues have also been found to be present on components of nematode ES products and somatic extracts and have been described in a number of helminths from *D. viviparus* (Haslam *et al*., 2000), *H. contortus* (Haslam *et al*., 1996; Vervelde *et al*., 2003) and *Toxocara canis* (Maizels and Page, 1990). N-linked glycans, in particular a glycan epitope termed 'Lewis^x' (Galβ1-4(Fucα1-3)GlcNAc), were discovered to be present in detergent extracts of *D. viviparus* (Haslam *et al*., 2000). The glycan structure termed 'Lewis^x' has been implicated in the promotion of a humoral immune response over a cellular response (Velupillai and Harn, 1994). In a vaccination trial of lambs against *H. contortus*, following immunisation with adult *H. contortus* ES products, specific antibodies were produced against glycan components of the ES products (Vervelde *et al*., 2003). Furthermore, the levels of serum IgA and IgG specific to ES antigens were seen to decrease if the ES antigens had been treated with sodium periodate prior to antibody probing in an ELISA (Vervelde *et al*., 2003), indicating that the structure of the glycans were disrupted by oxidation with sodium periodate. From this, Vervelde *et al*. (2003) concluded that ES glycan components in *H. contortus* were immunogenic and also induced a T-cell-dependent response, suggesting that that the glycan-specific immune response may contribute to protection against nematode infection.

Following on from this work, using stage-specific lectin binding, it was discovered that glycans with a high mannose component are present on the surface of *T. circumcincta* adults and sheathed L_3 (Hillrichs *et al*., 2012). Earlier work discovered a

31 kDa glycoprotein on the cuticle surface of *T. circumcincta* L_3 (McGillivery *et al*., 1989). In addition, an immunisation trial with this L_3 antigen showed that following a 40,000 L_3 bolus challenge, there was a significant reduction in both faecal egg counts and total worm counts in vaccinated lambs compared to unvaccinated controls (McGillivery *et al*., 1992). Antibodies specific to the antigen were detected in the serum from vaccinated lambs as early as 3 weeks after the start of the immunisation regime (McGillivery *et al*., 1992). Furthermore, an additional L_3-specific carbohydrate-larval antigen (CarLA), was found to be present on the cuticle surface of numerous nematode species, including *Trichostrongylus colubriformis, T. circumcincta*, *H. contortus*, *Nematodirus americanus* and *Cooperia curticei* (Harrison *et al*., 2003a; 2003b). Studies with *T. colubriformis* found that antibodies specific to CarLA were strongly associated with immune-mediated rejection of incoming L_3 (Harrison *et al*., 2003a; 2008). However, further research has revealed that there is variation in the CarLA antigens present on individual worms (Maass *et al*., 2009), which further suggests that carbohydrates on the surface of nematode parasites are recognised by the host immune system.

In this Chapter, the aim was to investigate the immunoreactivity of antigens present in extracts of the parasitic life-cycle stages of *T. circumcincta* using abomasal mucus and gastric lymph antibody probes generated from sheep which had been previously exposed to this parasite. As glycans are thought to have an important role in immunity against nematodes (Dell *et al*., 1999), this Chapter details investigation into the role of carbohydrate moieties in the generation of local immune responses against *T. circumcincta* infection. Glycosylation of antigens can influence epitope structure, so work presented here aimed to target antibodies directed against strucutural non-linear epitopes. To achieve this, sodium periodate was used to disrupt carbohydrate structures by cleaving hydroxyl groups without altering the structures of polypeptide chains (Woodward *et al*., 1985). For all immunological investigations, two kinds of antibody probes, generated from previously infected animals responding to a challenge infection, were to be used. Abomasal mucus was obtained *post mortem* from the abomasum of previously infected sheep and efferent gastric lymph was generated following lymphatic

cannulation (Smith *et al*., 1981). In the ovine gastrointestinal system, the efferent lymph duct drains all four ruminant stomachs (Smith *et al*., 1981). Efferent gastric lymph is a suitable medium to study local reactions in the abomasum, as the other three stomachs (reticulum, rumen and omasum) are immunologically inert and make minimal contributions to the changes occurring in the gastric lymph (Smith *et al*., 1981). Efferent gastric lymph can be collected through cannulation of the common gastric lymph duct, with a catheter fitted to allow collection of the gastric lymph into a sterile pouch (Halliday *et al*., 2007; Smith *et al*., 1981). A sub-sample of the lymph is taken daily and the remaining lymphatic outflow is re-infused into the sheep by a second catheter in the jugular vein (Halliday *et al*., 2007; Smith *et al*., 1981). The overall aim was to use antibody probes generated from the abomasal mucus and gastric lymph of sheep previously infected with *T. circumcincta* to determine if carbohydrate moieties on larval antigens have a role in antibody binding.

2.2 Materials and methods

2.2.1 Infection protocols for production of ovine abomasal mucus and gastric lymph

All experimental protocols involving the use of animals, outlined or summarized in this thesis, were approved by the Moredun Research Institute's (MRI) Experimental and Ethical Review Committee and authorised under the UK Home Office Animals (Scientific Procedures) Act 1986.

2.2.1.1 Infection protocol for production of abomasal mucus

A trial had been performed prior to the current study, in which the immune responses of yearling sheep, which had received an experimental trickle infection of *T. circumcincta* L_3, were compared to those responses of helminth-naïve yearlings (Halliday *et al.*, 2012; Knight *et al.*, 2011). Both groups of yearlings were administered a challenge dose of 50,000 L_3 and euthanased at 2 dpc. A summary of the experimental design which was used is illustrated in Figure 2.1. The project under which was this animal experiment was performed was entitled Combating Endemic Diseases of Farmed Animals (CEDFAS), and was funded by a BBSRC grant (BB/E018610/1) awarded to Professor D. Knox. All sheep used were Scotch Mules (Blackface ewe X Blue-faced Leicester ram), and were 8 months-old at the start of the trickle infection and 10 months-old at necropsy. The sheep were raised indoors to reduce the risk of helminth exposure until the start of the trickle infection.

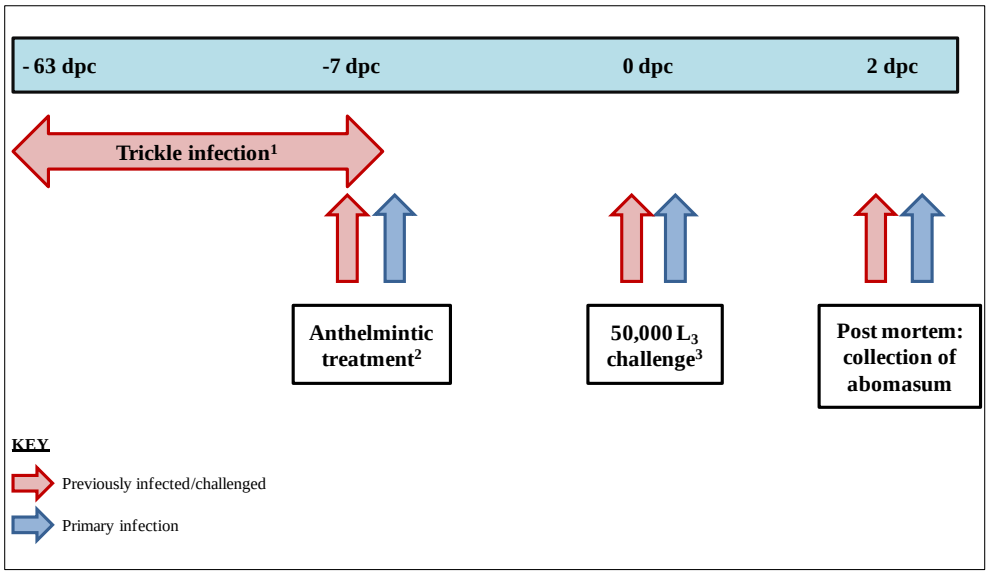

Figure 2.1 Design of experiments in sheep from which abomasal mucus was obtained.

[1]Trickle infection: 2,000 *T. circumcincta* L_3 three times per week for eight weeks. Trickle infection administered to previously infected/challenged group only (Red).
[2]Fenbendazole (5mg/kg) administered to both groups seven days before challenge.
[3]Bolus of 50,000 *T. circumcincta* L_3 challenge administered to both groups on day 0.

A group of lambs (*n*=4) were trickle-infected *per os* with 2,000 *T. circumcincta* L_3, three times per week for eight weeks (Figure 2.1, Red). Faecal worm egg counts (FWEC) were performed on samples taken from the lambs at the end of the trickle infection programme. Another group of lambs (*n*=2) had been kept in helminth-free conditions (Figure 2.1, Blue), and were not exposed to trickle infection. Both groups were administered with fenbendazole (5mg/kg) to remove any residual worms from the animals before a bolus challenge. Seven days later, both groups were challenged *per os* with a single bolus dose of 50,000 *T. circumcincta* L_3. All animals were killed by captive bolt and exsanguinated at 2 dpc, the abomasum removed and contents emptied before cutting along the greater and lesser curvatures. Each half of the abomasum was washed in a bucket of physiological saline (kept at 37°C) to remove any remaining contents and debris. The entire luminal surface of abomasal tissue was dabbed with sterile gauze (9.5 cm x 10 cm) pre-soaked in 3ml sterile phosphate-buffered saline (PBS), pH 7.2. To retrieve mucus, the gauze was inserted into the barrel of a 20 ml

syringe and the contents emptied into a sterile 25 ml tube. The mucus was then aliquoted to 1 ml volumes and centrifuged at 3000 *g* for 5 min. The supernatant was removed, aliquoted into fresh 1.5 ml tubes and stored at -20°C. Worm counts were not performed as the main focus of the experiment was to use the abomasal tissue as explants.

2.2.1.2 Infection protocol for collection of efferent gastric lymph

A series of large-scale experiments had been performed prior to the current study on a project funded through the Veterinary Training Research Initiative (VTRI) (http://www.vet.ed.ac.uk/vtri) from the Department for Environment Food and Rural Affairs, BBSRC and by the Scottish Executive Environment and Rural Affairs Department. Access was given to the biobank of samples by Dr. W. D. Smith (Moredun Research Institute). Here, efferent gastric lymph was collected following cannulation of the common gastric lymph duct of sheep experimentally infected with *T. circumcincta* (Halliday *et al*., 2007). All sheep used in the experiments were Suffolk-Dorset or Scottish Blackface-Leicester cross. There were two groups: (1) trickle-infected/challenged and (2) primary infected. For both groups, there were two sub-groups divided according to their age: (1) yearlings and (2) <6-month-old lambs. All sheep had been reared under conditions designed to exclude accidental infection with helminths. Cannulation of the common gastric lymph duct (described in Smith *et al*., 1981) was performed to allow collection of efferent gastric lymph from selected sheep through a specific time frame. The sheep underwent surgicial procedures to have a primary catheter fitted to the common gastric lymph duct (near the abomasum site) with a secondary catheter fitted to the jugular vein (Halliday *et al*., 2007; Smith *et al*., 1981). Cannulated sheep were housed individually into small pens and lymph was collected into a sterile, heparinised urine drainage bag supported by a harness (Smith *et al*., 1981). Eeach day bags were weighed to estimate the lymph flow rate and a 10-20 mL lymph sample was collected into heparinised tubes (Halliday *et al*., 2007). The bag contents were then re-infused intravenously into the jugular vein through the secondary catheter under gravity (Halliday *et al*., 2007; Smith *et al*., 1981).

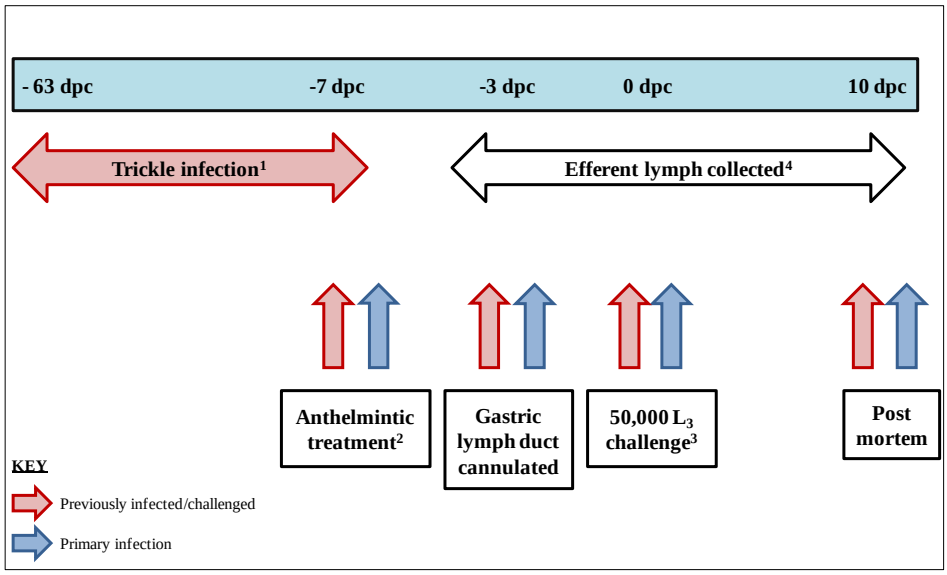

Figure 2.2 Design of experiments in sheep from which efferent gastric lymph was obtained

[1]Trickle infection: 2,000 *T. circumcincta* L_3 three times per week for eight weeks. Trickle infection administered to previously infected/challenged group only (Red).
[2]Levamisole (7.5mg/kg) administered to both groups seven days before challenge.
[3]Bolus of 50,000 *T. circumcincta* L_3 challenge administered to both groups on day 0.
[4]Common gastric lymph duct cannulated at -3 dpc in both groups. Efferent gastric lymph collected from -2 to 10 dpc.

The 'trickle-infected/challenged' sheep were infected with 2,000 *T. circumcincta* L_3, three times per week for an eight-week period (Figure 2.2, Red). A second group was kept helminth-free and did not receive the trickle infection (Figure 2.2, Blue). To remove residual worms, all sheep were treated with levamisole (dose: 7.5mg/kg), seven days prior to the bolus challenge. The common gastric lymph ducts of all animals were surgically cannulated and efferent lymph collected daily from -2 dpc to 10 dpc. Three days after cannulation, all groups were given an oral challenge dose of 50,000 *T. circumcincta* L_3. The 'trickle-infected/challenged' sheep were killed on day 10 post-challenge and abomasal adult worm counts were performed using established methods (Coop *et al.*, 1977). Gastric efferent lymph samples collected from the challenge-only sheep from -3 dpc to 0 dpc, were referred to as 'helminth naïve'.

Project name	Sample	Infection status	Abbreviation
CEDFAS	Abomasal mucus	Trickle infection/challenge Primary infection (challenge only)	M-PI M-CO
VTRI	Efferent gastric lymph	Trickle infection/challenge Primary infection (challenge only) Helminth-naive	GL-PI GL-CO GL-N

Table 2.1 Summary and abbreviations for samples used in immunoreactivity investigations

Key: M = Abomasal mucus; GL = Efferent gastric lymph; PI = Previously infected by a trickle infection/challenge; CO = Primary infection with a single bolus challenge; N = Helminth-free naive sheep.

Lymphocytes were isolated from the efferent gastric lymph through fluorescence-activated cell sorting (FACS) using cell specific markers (see Halliday *et al*., 2009; 2010). Also, large cells or lymphoblasts were determined as those with a diameter of >9mm when measured by Coulter Counter, with small lymphocytes represented as those with a diameter of 3-9mm (Halliday *et al*., 2010).

2.2.2 Recovery of L_4 from the abomasum

For the production of L_4, six helminth-free 6-month-old Scotch Mule (Blackface ewe X Blue-faced Leicester ram) lambs were infected orally with 100,000 *T. circumcincta* L_3 and euthanised seven days later to recover the 7 day-old L_4. At post mortem, the abomasum was removed and the contents retrieved as described in Section 2.2.1.1. To remove any remaining debris, the surface of the abomasum was washed briefly in sterile physiological saline. The abomasum was pinned to a polystyrene block so that the abomasal folds were suspended into the fluid-filled funnel, allowing larvae to come out from the mucosa into the funnel. The pinned abomasum was floated on top of a 5 l funnel, filled with sterile physiological saline (NaCl:H_20 solution), pH 7.4, at 37°C using an adapted Baermann-style technique (Knox and Jones, 1990) Larvae that gravitated to the bottom of tubing attached to the funnel were retrieved after 2, 4 and 6 h. L_4 were transferred to 50 ml conical tubes and centrifuged at 50 *g* for 5 min to aid settling. The supernatant was aspirated, and the L_4 which had settled at the bottom of the tubes were kept and pooled.

2.2.3 *In vitro* culture of L_4 to collect excretory/secretory products

L_4 (collected as described in section 2.2.2) were washed three times in 20 ml of sterile PBS, pH 7.4, incubated in a waterbath at 37°C for 20 min, centrifuged at 50 *g* for 5 min between washes and the supernatant aspirated. Following washing, the L_4 were resuspended in 20 ml RPMI-based 'nematode culture medium' (for recipe see Appendix 1), incubated at 37°C for 20 min, before being centrifuged at 50 *g* for 5 min to re-pellet the L_4. Supernatants were removed and the L_4 pellets suspended in 40 ml fresh nematode culture medium before being transferred to 75cm^2 cell culture flasks with vented cap (Corning Incorporated) and incubated at 37°C, 5% CO_2 for 24 h. Following the initial 24 h incubation period, the contents of larvae/media were transferred to a 50 ml conical tube and centrifuged at 50 *g* for 5 min at 4°C. The supernatants were removed under aseptic conditions and transferred to fresh 50 ml tubes, on ice. The L_4 pellets were resuspended in 40 ml of fresh nematode culture medium and incubated at 37°C, 5% CO_2 for another 24 h. Supernatants from both time-points (24 h and 48 h cultures) were filtered through 0.45µM syringe filters, transferred to Amicon Ultra-15 centrifugal filter units (Millipore, UFC901096) and centrifuged at 2000 *g* for 20 min at 4°C to concentrate excretory/secretory ('ES') products. The ES products were concentrated 15-fold to approximately 1 ml volume, and buffer exchanged by washing and centrifuging three times in PBS to remove media contents, then centrifuged at 2000 *g* for 20 min at 4°C. Once buffer was exchanged, the ES products (approximately 1 ml) were dispensed into 100 µl aliquots and stored at -80°C.

2.2.4 Preparation of L_3 somatic antigen

PBS-soluble somatic extracts were prepared from *T. circumcincta* L_3 by crushing a 1ml aliquot of snap-frozen worms (~1 million) with a pestle and mortar (pre-chilled at -80°C) in the presence of liquid nitrogen. After 10 min of grinding, 5 ml PBS were added and, once no particulate material was remaining, a further 5 ml of PBS were added and the extract mixed thoroughly. The mixture was aliquoted into 1 ml volumes, centrifuged at

9000 *g* for 5 min at 4°C to pellet insoluble material and the supernatant retained as 500 µl aliquots, stored at -80°C. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Scientific Ltd). Briefly, 11 bovine serum albumin (BSA) standards were prepared by diluting a stock solution of BSA in PBS to concentrations ranging from 0-2 mg/ml. L_3 somatic extracts were diluted 1:2, 1:5, and 1:10 in PBS and 25 µl of sample or standard were dispensed in triplicate into individual wells of a 96-well Microlon® ELISA plate. The BCA reagent was prepared by mixing the kit reagents A and B in the ratio of 50:1, respectively and 200 µl were added to each well. Plates were incubated at 37°C for 30 min and absorbance measured at 562nm on a 96-well plate reader. Using the BSA standards, reference standard curves were plotted and protein concentrations of the somatic extracts determined.

2.2.5 Preparation of L_4 and adult somatic extracts

L_4 and adult somatic extract were kindly donated by Dr. Rachael Baker (MRI) and had been prepared by the following method. An aliquot of ~1 million *T. circumcincta* L_4 or adults, previously snap-frozen in liquid nitrogen, was homogenized in 2 ml ribolyser tubes (Q-BIOgene Lysing matrix D ceramic beads, Irvine, USA) for 45 secs in PBS containing 1mM EDTA and 1mM PMSF. Following centrifugation at 20000 *g* for 20 min, the supernatants were retained. The protein concentrations of somatic extracts were determined by the BCA assay (Pierce, Thermo Scientific) as described in Section 2.2.4.

2.2.6 SDS-PAGE

The protein profiles of L_3, L_4 and adult somatic extracts and L_4 ES products were investigated by 1-dimensional (1-D) gel electrophoresis. Using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) the proteins were separated, under reducing conditions, on 4-12% gradient gels using the NuPAGE Novex Bis-Tris

Mini Gel System (Invitrogen Ltd, UK), according the manufacturer's recommendations. For all experiments, ~10 µg of protein were loaded per well and electrophoresis performed using MES SDS running buffer with antioxidant (Invitrogen Ltd, UK). Parasite extracts or ES products were added to SDS sample buffer and sample reducing agent (Invitrogen Ltd, UK) and incubated at 70°C for 10 min to reduce and denature the proteins. Each sample was then applied to a single well (approximately 10-20 µl of sample containing 10 µg of protein). The apparatus was connected to a power supply and run at 200V for approximately 35 min or until the dye front reached the bottom of the gel. Once run, one lane of each extract was stained with colloidal Coomassie stain (Simply Blue Stain, Invitrogen, UK) for 60 min at room temperature and then rinsed in distilled water until protein bands were visible against a clear background to allow estimation of the molecular weight of protein bands in the different extracts.

2.2.7 Western blotting

SDS-PAGE (Section 2.2.6) was performed on each parasite extract or ES batch, and the separated proteins transferred by electroblotting onto a 0.45 µM nitrocellulose membrane using the X-Cell-II Blot Module (Invitrogen Ltd, UK), according to the manufacturer's instructions. Successful transfer of the proteins was confirmed by staining of the nitrocellulose membrane in Ponceau stain (0.1% in 5% acetic acid) (Appendix 1) before destaining in distilled water. Once electroblotted, the nitrocellulose membrane was incubated in blocking buffer, TNTT (10mM Tris, 0.5M NaCl, 0.05% (v/v) Tween-20, 0.01% (w/v) thiomersal, pH 7.4), to block non-specific binding sites for proteins. After an overnight incubation at 4°C, membranes were washed three times in fresh TNTT, 10 min per wash. The lanes were separated and then incubated with primary antibody (abomasal mucus or gastric lymph diluted 1:5 and 1:100 in TNTT, respectively) from previously infected/challenged or challenged only sheep for 2 h at room temperature. Membranes were then re-washed in TNTT and incubated with the appropriate secondary antibody according to the specific antibody isotype under investigation (Table 2.2) for 1 h at room temperature. The antibodies were diluted to the

required concentration in TNTT. For IgA investigations, a conjugated tertiary antibody was required (Table 2.2) and incubations were conducted for 1 h at room temperature following washes in TNTT. After the application of the final HRP-conjugated antibody (*i.e.* IgG, secondary; IgA, tertiary), strips were rewashed in TNTT, then incubated in 3,3'-diaminobenzidine (DAB, Sigma Fast™, Sigma-Aldrich, UK) for colour development of the substrate for approximately 5 min, then washed repeatedly in distilled water and left to dry.

Isotype	**Secondary antibody**			**Tertiary antibody**		
	Description	**Manufacturer**	**Dilution**	**Description**	**Manufacturer**	**Dilution**
IgA	Mouse monoclonal anti-bovine/ovine IgA	Serotec, MCA628	1 in 250	Polyclonal rabbit anti-mouse immunoglobulins HRP- conjugated	Dako, P0260	1 in 1000
IgG	Mouse monoclonal anti-goat/sheep IgG HRP-conjugated	Sigma, A0452	1 in 1000	N/A	N/A	N/A

Table 2.2 Antibodies used for antigen-specific ELISAs and immunoblots.

2.2.7.1 Periodate treatment of immunoblots

Where the analysis of carbohydrate binding was being investigated, immunoblots were treated with sodium periodate prior to antibody incubations. Sodium periodate degrades carbohydrate moieties through oxidation of hexose sugar rings to amine-reactive aldehyde groups, resulting in the disruption of carbohydrate epitopes present (Woodward *et al.*, 1985). The optimal concentration of sodium periodate was determined to ensure that there was no degradation of the integrity of the polypeptide backbone of the proteins in the antigen preparation. Thus, 5 µg of L_3 somatic extract were separated in individual wells of a 4-12% NuPAGE gel under reducing conditions. Separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was soaked in methanol for 30 sec, rinsed in distilled water and then soaked in transfer buffer before assembled into the X-Cell-II Blot Module. Proteins

were transferred to the PVDF membrane under the same conditions as detailed for nitrocellulose membranes in section 2.2.7. Successful transfer of proteins to the membrane was confirmed with Ponceau stain as above, and the blot was cut into individual lanes and rinsed in distilled water before treatment for disruption of carbohydrate. For the initial optimisation, strips were washed in 50mM sodium acetate, pH 4.5, twice for 20 min per wash. Each strip was incubated with a different concentration of sodium meta-periodate (range 0–50mM) in 50mM sodium acetate for 1 h at room temperature in the dark. Lanes were re-washed in 50mM sodium acetate twice, then washed twice in TNTT, for 10 min per wash. The strips were incubated in 50mM sodium borohydride/TNTT for 30 min at room temperature, before three final washes in TNTT. To detect the pattern of protein banding in the samples, all the strips were stained with Simply Blue stain (Invitrogen Ltd, UK) for 10 min at room temperature. After destaining in 40% methanol to remove background staining, protein profiles were compared across the range of sodium periodate concentrations. Subsequently, the following protocol was used for periodate treatment of immunoblots: SDS-PAGE, electroblotting and blocking were performed as detailed in sections 2.2.6 and 2.2.7. Membrane strips were washed twice in 50mM sodium acetate, pH 4.5 for 20 min, then incubated in 50mM sodium periodate/50mM sodium acetate in the dark for 1 h at room temperature. The strips were re-washed twice in 50mM sodium acetate and then washed twice in TNTT, for 10 min per wash. Blots were incubated in 50mM sodium borohydride/TNTT for 30 min at room temperature. After three additional TNTT washes, the blots underwent a series of antibody incubations as required for the isotype under investigation (described in section 2.2.7).

2.2.8 Detection of specific antibodies by ELISA

For quantitative assessment of antigen-specific antibody responses, microtitre plates (Greiner Bio-one, flat-bottom high binding) were coated with 50 µl of 5µg/ml *T. circumcincta* extracts from L_3, L_4 and adult homogenates and L_4 ES products, in 50mM sodium bicarbonate, pH 9.6, and incubated at 4°C overnight. Plates were washed six

times with PBS containing 0.05% (v/v) Tween-20 (PBST) with an automated plate washer (BioTek ELX405). Plates were incubated with blocking buffer (10% soya milk powder (Infasoy, Cow and Gate) in PBST) at 200µl per well for 2 h at room temperature to prevent non-specific binding. After re-washing, plates were incubated with 50 µl of primary antibody (abomasal mucus or efferent gastric lymph, diluted at 1:5 and 1:20 in TNTT, respectively), and incubated for 2 h at room temperature. After washing, 50 µl of secondary antibody (see Table 2.2 for antibody incubations) diluted in TNTT were added and incubated for 1 h at room temperature. When a tertiary antibody was required to detect IgA binding, following washing, 50 µl of tertiary antibody, diluted to the appropriate concentration in TNTT, were added to each well and incubated for 1 h at room temperature. After the final antibody incubation step, the plates were washed six times in PBST and then 50 µl *O*-phenylenediamine dihydrochloride (OPD) substrate (Sigma Fast™, Sigma-Aldrich, UK) were added. Plates were incubated in darkness for 20 min, the reaction was stopped by addition of 25 µl 2.5M H_2SO_4 per well and the optical density (OD) read at 492nm on a spectrometer. All tests were conducted in triplicate on each plate and each plate was repeated on two independent occasions. Negative controls omitting the primary antibody incubation step were included on each plate. A pool of efferent gastric lymph collected from VTRI 'previously infected/challenged' sheep at the time-point corresponding to the peak total IgA concentration, 6-10 dpc (Halliday *et al*., 2007), was used as a positive reference sample on all plates. To control plate-to-plate variation, the absorbance value from the blank wells (wash buffer only) was subtracted from raw absorbance values before analysis.

2.2.8.1 Periodate treatment of antigens for ELISA

For optimization, L_3 somatic extract was treated with a range (0-100mM) of sodium periodate concentrations in 50mM sodium acetate, pH 4.5. Ten µg of L_3 somatic extract were added to a 1.5 ml tube, with 50 µl of appropriate concentration of sodium periodate and incubated for 1 h in the dark at room temperature. Solutions were centrifuged at 10000 *g* for 10 min, and the supernatants from each preparation separated by

electrophoresis using a NuPAGE SDS gel (Section 2.2.6). The gel was stained with Simply Blue stain (Invitrogen Ltd, UK) and comparisons made across the range of sodium periodate concentrations with regards to the level of protein degradation. To investigate the contribution that carbohydrate moieties made towards generating local antibody responses, L_3 and L_4 antigen preparations were pre-treated with sodium periodate in solution before ELISA analysis. Antigen-specific IgA and IgG antibody binding to L_3 somatic, L_4 somatic and L_4 ES antigens were treated with sodium periodate was compared to binding to non-periodate-treated antigens. ELISAs were carried out as described in section 2.2.8, but the following additional steps were included after sample addition. Plates were washed six times with PBST, then incubated with 10mM sodium periodate/50mM sodium acetate in a final volume of 100 µl/well. Plates were incubated at 37℃ for 1 h in the dark. Plates were re-washed twice with 50mM sodium acetate and then twice with TNTT (200µl/well). To stop the oxidative activity of sodium periodate, 50mM sodium borohydride/TNTT were added to each well (100µl/well) and incubated at 37°C for 30 min. Following the sodium borohydride incubation, plates were re-washed six times with PBST, and were processed for antibody incubations as required for the isotype under investigation

2.2.9 Local immune response to L_3 antigens by antibodies in efferent gastric lymph

To investigate antibody responses in efferent gastric lymph to L_3 antigens over the duration of a 50,000 L_3 bolus challenge infection (VTRI experimental trial -2 to 10 dpc), ELISAs were conducted using samples from two animals. The animals selected (#A295 and #A1008) were chosen as they demonstrated high total IgA concentrations (mg/ml) in efferent gastric lymph following challenge with L_3; IgA titres were 52.9% and 33% above the average of 3.65mg/ml at 8 dpc, with readings of 4.92mg/ml and 4.29mg/ml, respectively. IgA and IgG responses to L_3 antigens were analysed from days -2 to 10 post-challenge and ELISAs were conducted as detailed in Section 2.2.8. To investigate the antibody response to L_3 somatic antigens during the peak period of total IgA

concentration in efferent gastric lymph, 6-10 dpc, and immunoblots using samples collected from individual sheep in this time period were used. Immunoblots were conducted as detailed in Section 2.2.7. All samples were investigated for IgA and IgG reactivity against L_3 somatic extracts. Separate immunoblots were treated with sodium periodate (Section 2.2.7.1) to assess the contribution that carbohydrate moieties made towards generating the local antibody response against L_3.

2.2.10 Statistical analysis

In the antigen-specific ELISAs (Section 2.3.1), the data from each ELISA set were tested for normality of data through a residual plot of the individual OD values. The probability plot of the residuals showed normal probability. The differences between the mean OD values were statistically evaluated by the parametric one-way ANOVA test adjusted for the post hoc analysis, Fisher's least significant difference. To allow comparisons to be made on the effect of sodium periodate treatment of antigens towards the level of local immunoreactivity in the periodate antigen-specific ELISAs (Section 2.3.2), differences between the mean OD values obtained from periodate-treated and untreated antigen samples were statistically evaluated by the non-parametric Wilcoxon-Mann-Whitney U test (SPSS version 19). For all statistical analysis tests the statistical significance was set at $p\text{-value}<0.05$.

2.3 Results

2.3.1 Isotype-specific antibody responses to *T. circumcincta* larval antigens

ELISAs were used to investigate antibody isotypes present in abomasal mucus and gastric lymph that bound *T. circumcincta* larval antigens. Both L_3-specific IgA and IgG were detected in abomasal mucus obtained from previously infected/challenged sheep (Figure 2.3). Mucus IgA activity against L_3 somatic antigens was significantly higher ($p<0.001$) in previously infected/challenged sheep compared to sheep given a single challenge, with the percentage positivity being 100% and 23%, respectively (Figure 2.3). Likewise, the level of IgG binding to L_3 antigens was significantly higher in the abomasal mucus from previously infected/challenged sheep compared to sheep given a single challenge and helminth-naïve sheep ($p=0.008$). IgG binding to L_3 antigens was detected in the efferent gastric lymph sample from helminth-naïve sheep (Figure 2.3; 17% positivity).

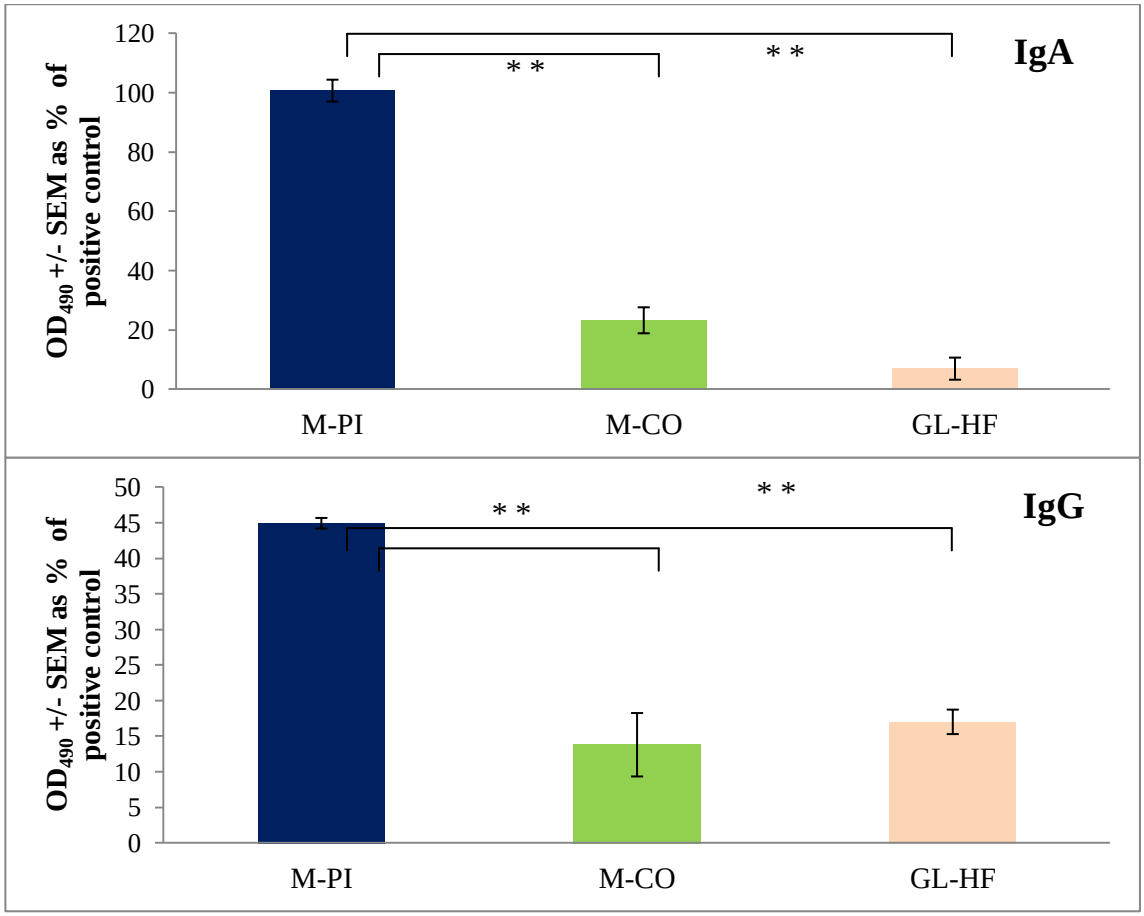

Figure 2.3 Mucus IgA and IgG responses in previously infected and primary infected sheep to L_3 somatic antigens.

'OD' represents optical density value at 490nm measured in an ELISA to determine mucus IgA and IgG activity to L_3 somatic antigens. Group "M-PI" represents abomasal mucus pooled from previously infected sheep and group "M-CO" represents abomasal mucus pooled from sheep given a single infection. In both groups the sheep were given a single bolus challenge of 50,000 *T. circumcincta* L_3 and necropsied 2 dpc to collect abomasal mucus. Group "GL-HF" represents pooled efferent gastric lymph from helminth-naïve sheep and was used as a 'negative control'. The positive control was pooled efferent gastric lymph collected over 6-10 dpc from previously infected/challenged sheep. The mean OD (± standard error of mean (SEM)) results are expressed as a percentage of the positive control. '**' denotes statistically significant differences at $p<0.01$ between the mean OD values of the M-PI group and the groups M-CO and GL-HF.

Abomasal mucus IgA and IgG activity against L_4 somatic antigens was detected in samples collected from previously infected/challenged sheep (Figure 2.4). Levels of mucosal antibodies specific to L_4 somatic antigens were significantly higher for both

isotypes in the previously infected/challenged sheep ($p<0.01$) compared to the sheep given a single challenge and helminth-naïve sheep (Figure 2.4).

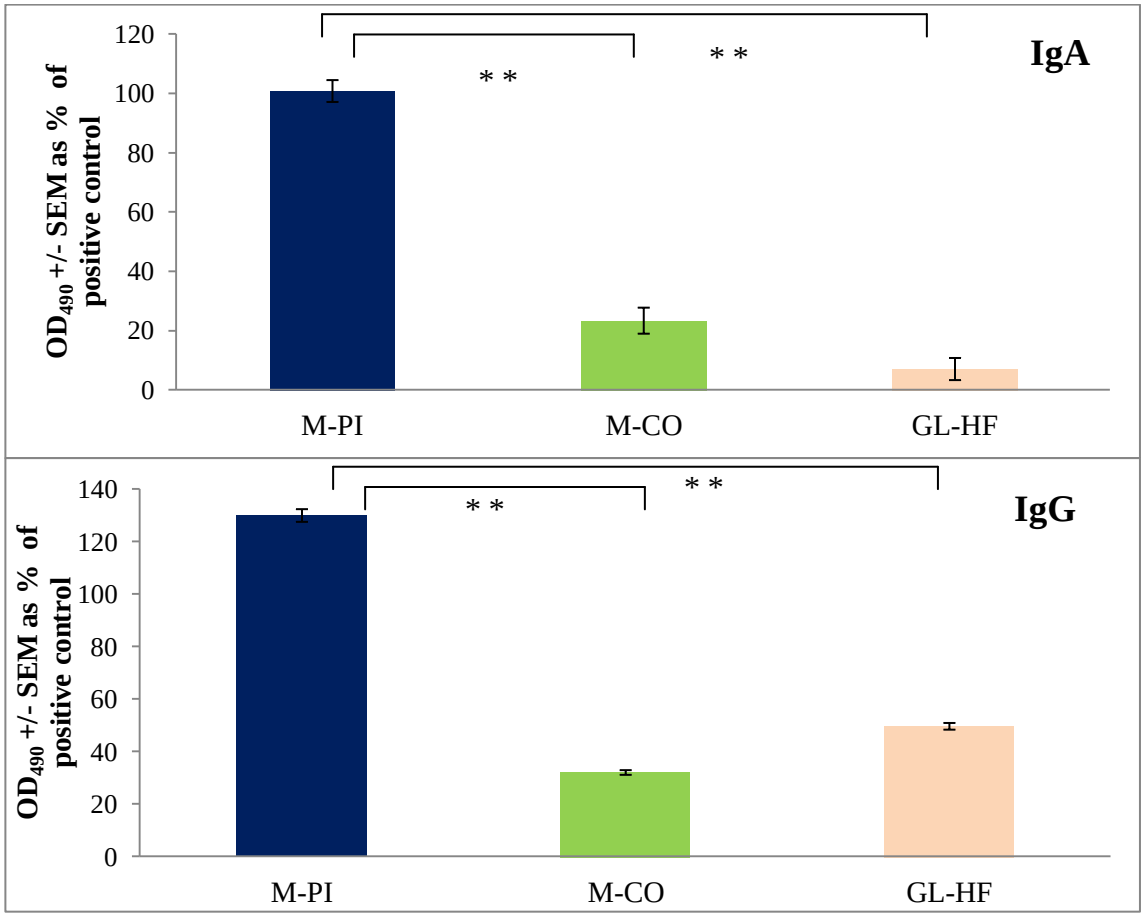

Figure 2.4 Mucus IgA and IgG responses in previously infected and primary infected sheep to L_4 somatic antigens.

'OD' represents optical density value at 490nm measured in an ELISA to determine mucus IgA and IgG activity to L_4 somatic antigens. Group "M-PI" represents abomasal mucus pooled from previously infected sheep and group "M-CO" represents abomasal mucus pooled from sheep given a single infection. In both groups the sheep were given a single bolus challenge of 50,000 *T. circumcincta* L_3 and necropsied 2 dpc to collect abomasal mucus. Group "GL-HF" represents pooled efferent gastric lymph from helminth-naïve sheep and was used as a 'negative control'. The positive control was pooled efferent gastric lymph collected over 6-10 dpc from previously infected/challenged sheep. The mean OD (± standard error of mean (SEM)) results are expressed as a percentage of the positive control. '**' denotes statistically significant differences at $p<0.01$ between the mean OD values of the M-PI group and the groups M-CO and GL-HF.

L_4 ES specific-IgA was detected in the abomasal mucus obtained from previously infected sheep at 2 dpc (Figure 2.5).

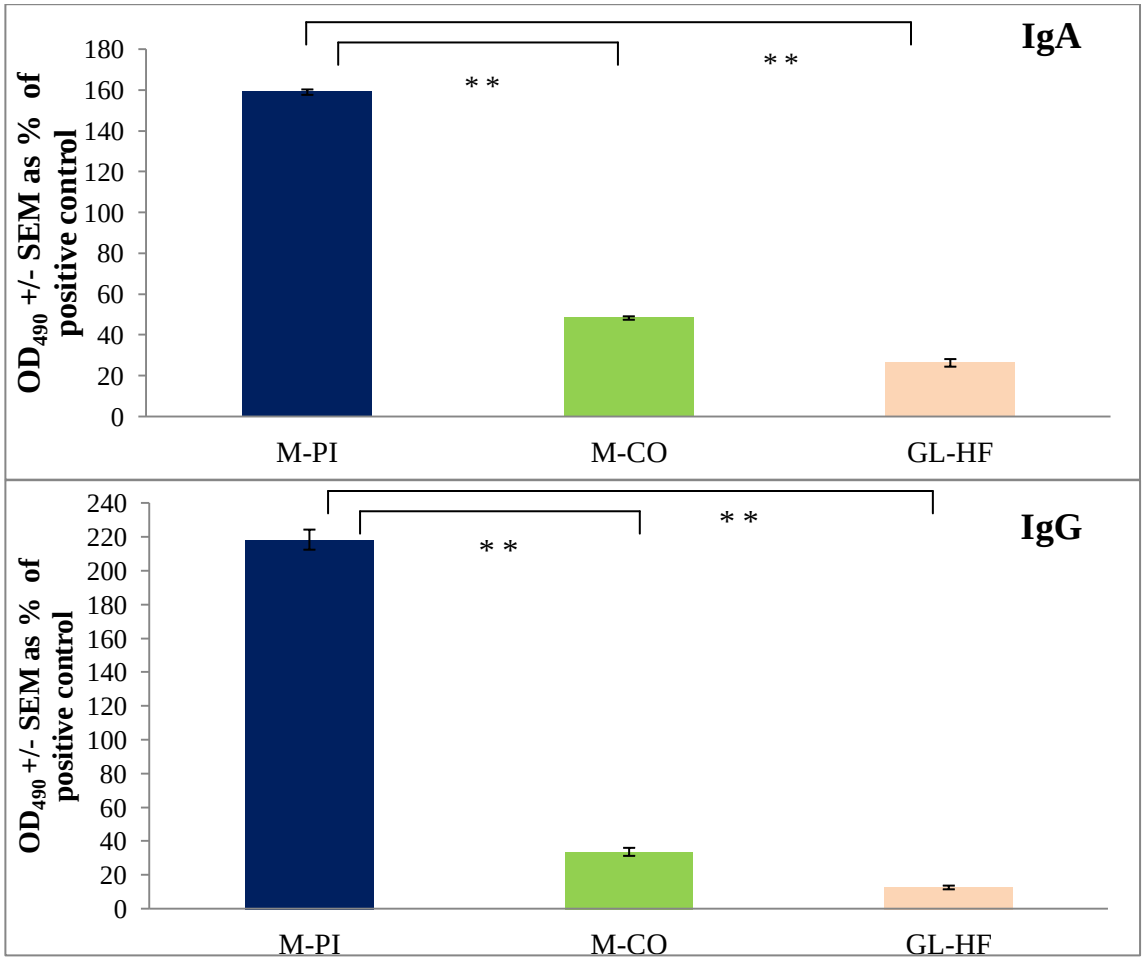

Figure 2.5 Mucus IgA and IgG responses in previously infected and primary infected sheep to L_4 ES antigens.

'OD' represents optical density value at 490nm measured in an ELISA to determine mucus IgA and IgG activity to L_4 ES antigens. Group "M-PI" represents abomasal mucus pooled from previously infected sheep and group "M-CO" represents abomasal mucus pooled from sheep given a single infection. In both groups the sheep were given a single bolus challenge of 50,000 *T. circumcincta* L_3 and necropsied 2 dpc to collect abomasal mucus. Group "GL-HF" represents pooled efferent gastric lymph from helminth-naïve sheep and was used as a 'negative control'. The positive control was pooled efferent gastric lymph collected over 6-10 dpc from previously infected/challenged sheep. The mean OD (± standard error of mean (SEM)) results are expressed as a percentage of the positive control. '**' denotes statistically significant differences at $p<0.01$ between the mean OD values of the M-PI group and the groups M-CO and GL-HF.

The levels of IgA binding to L_4 ES antigens was significantly higher ($p<0.001$) in previously infected sheep in comparison to sheep given a single challenge (Figure 2.5). Likewise, IgG bound to L_4 ES antigens and the level of binding detected was higher in

the previously infected sheep compared to the mucus from sheep given a single challenge (Figure 2.5).

2.3.2 Contribution of glycan to the antigen-specific antibody response in gastric lymph

To determine if glycans on larval antigens are important in immune responses to *T. circumcincta*, sodium periodate was used to disrupt the carbohydrate moieties. Initial experiments determined the effects of increasing sodium periodate concentrations on the protein profile in the *T. circumcinta* preparations. The optimal sodium periodate concentration for use in antigen-specific ELISAs was determined. The protein profiles of L_3 somatic extract after treatment in solution with a range of sodium periodate concentrations are shown in Figure 2.6. Treatment of the native L_3 antigen preparation with 10mM sodium periodate (Figure 2.6, Lane 2) did not affect the electrophoretic profile of the proteins in the extract, as the profile is the same as the extract incubated without sodium periodate (Figure 2.6, Lane 1). Incubation with sodium periodate at concentrations of 20mM and above altered the protein profile indicating degradation of proteins in the extract (Figure 2.6, Lanes 3-5). The optimal concentration for sodium periodate treatment of parasite antigens in the ELISA investigations was thus determined to be 10mM.

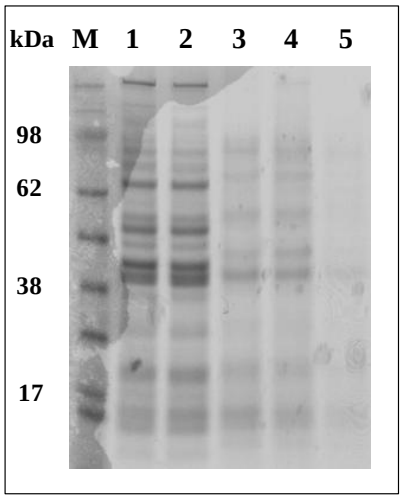

Figure 2.6 Protein profiles of *T. circumcincta* L_3 somatic extract following incubation of native antigen preparation with a concentration range of sodium periodate in solution.

Lane 1, No sodium periodate. Lane 2, 10mM sodium periodate. Lane 3, 20mM sodium periodate. Lane 4, 50mM sodium periodate. Lane 5, 100mM sodium periodate. All samples of L_3 somatic extracts (5µg) were incubated in solution with sodium periodate, and the supernatant retained and subjected to electrophoresis under reducing conditions. Protein was detected by Simply Blue stain. Lane M denotes molecular weight standards given in kDa.

To investigate the effect of pre-treatment with sodium periodate on the binding of sheep IgA and IgG to larval antigens, antigen-coated ELISA plates were treated with 10mM sodium periodate then processed for antibody probing. An equivalent number of antigen samples were not treated with sodium periodate prior to antibody probing to allow comparison. The results from the L_3 antigen-specific ELISA to assess the level of antibody binding with periodate-treated L_3 antigens and untreated L_3 antigens are presented as a percentage of the positive control (Figure 2.7).

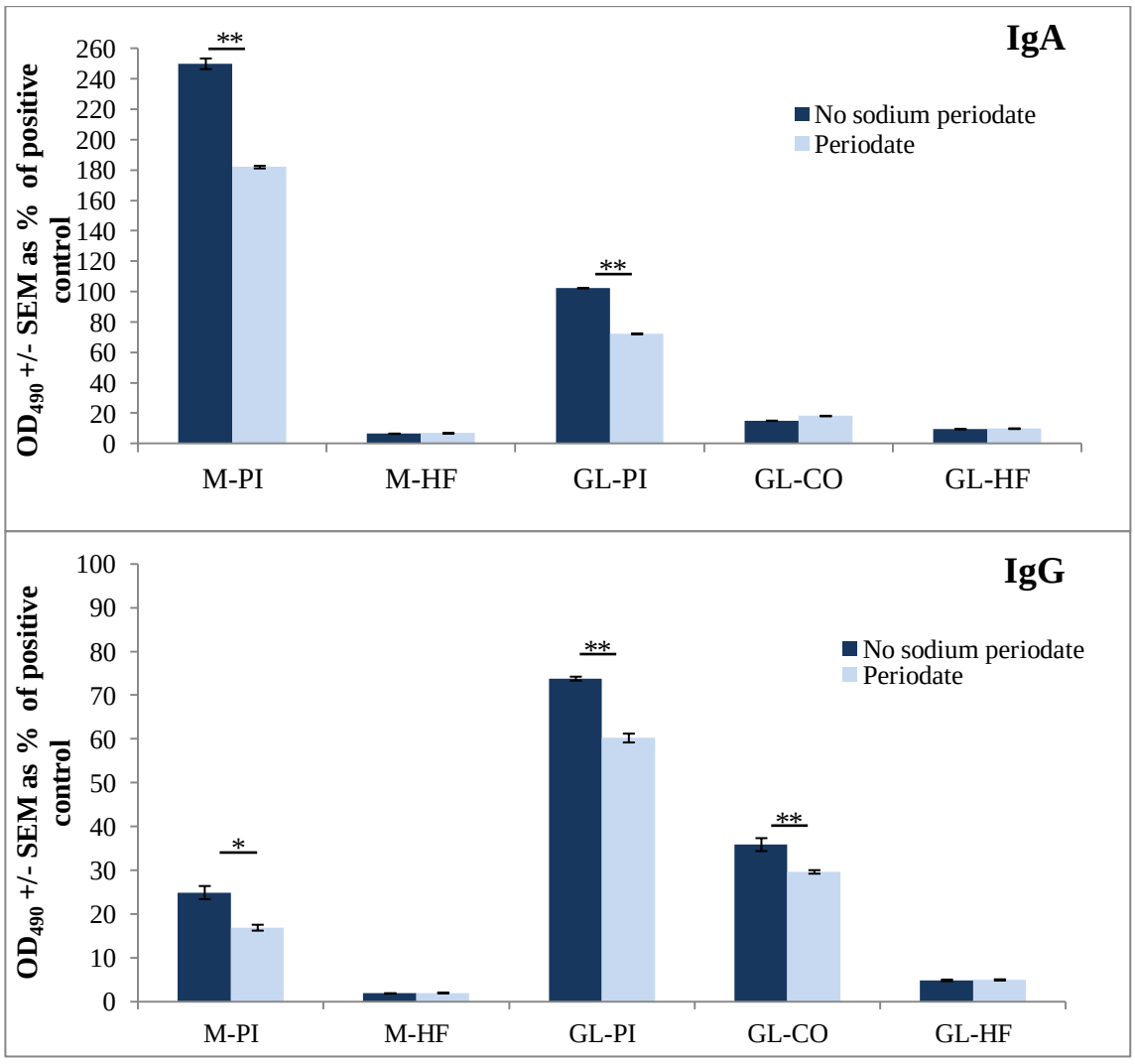

Figure 2.7 Effect of sodium periodate treatment of L_3 somatic extract on IgA and IgG antibody binding to L_3 somatic antigens.

The mean OD (± SEM) results are expressed as percentage of the positive control. The positive control was efferent gastric lymph pooled from previously infected/challenged sheep over 6-10 dpc. Group "M-PI", abomasal mucus pooled from previously infected sheep. Group "GL-PI", efferent gastric lymph from previously infected sheep. Group "GL-CO", efferent gastric lymph sheep given a single infection. In all groups the sheep were given a single bolus challenge of 50,000 *T. circumcincta* L_3. Group "M-HF", abomasal mucus pooled from helminth-naïve sheep. In the mucus groups, sheep were necropsied at 2 dpc to collect abomasal mucus. The gastric lymph samples were collected at 8 dpc. '**' and '*' denotes statistical significance ($p<0.01$, $p<0.05$) following comparison of the mean OD values between treated and untreated samples. For all groups, $n = 6$.

The ELISA results indicated that sodium periodate treatment of antigens resulted in a reduction in antibody binding in some of the groups. The reductions in the level of both IgA and IgG binding in the previously infected sheep were statistically significant ($p=0.004$) (Figure 2.7). The level of IgA binding in sheep given a single infection was not significantly affected by sodium periodate treatment of L_3 antigens. In contrast, the level of IgG binding in gastric lymph from sheep given a single infection was significantly reduced ($p=0.004$) following periodate treatment of L_3 antigens (Figure 2.7, IgG graph).

Following sodium periodate treatment of L_4 somatic antigens, the level of IgA binding in abomasal mucus and gastric lymph samples from previously infected sheep was significantly reduced ($p=0.003$) (Figure 2.8, IgA graph). The level of IgA binding detected in gastric lymph samples from sheep given a single infection was not affected by sodium periodate treatment of L_4 somatic antigens. Periodate treatment of L_4 antigens resulted in a significant reduction in the level of IgG binding in abomasal mucus and gastric lymph samples from previously infected and sheep given a single infection (Figure 2.8).

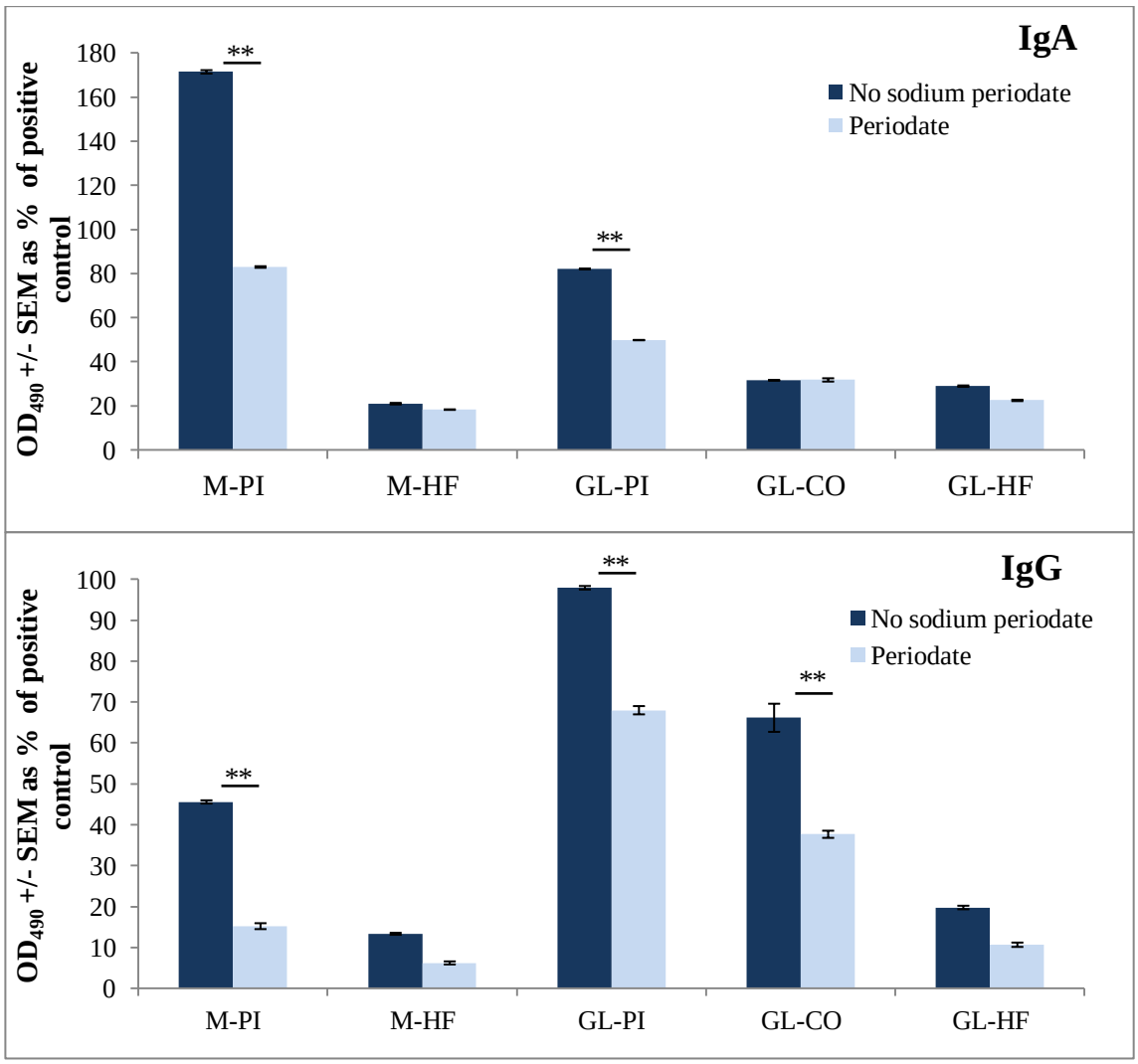

Figure 2.8 Effect of sodium periodate treatment of L_4 somatic extract on IgA and IgG antibody binding to L_4 somatic antigens.

The mean OD (± SEM) results are expressed as percentage of the positive control. The positive control was efferent gastric lymph pooled from previously infected/challenged sheep over 6-10 dpc. Group "M-PI", abomasal mucus pooled from previously infected sheep. Group "GL-PI", efferent gastric lymph from previously infected sheep. Group "GL-CO", efferent gastric lymph from sheep given a single infection. In all groups the sheep were given a single bolus challenge of 50,000 *T. circumcincta* L_3. Group "M-HF", abomasal mucus pooled from helminth-naïve sheep. In the mucus groups, sheep were necropsied at 2 dpc to collect abomasal mucus. The gastric lymph samples were collected at 8 dpc. '**' denotes statistical significance ($p<0.01$) following comparison of the mean OD values between treated and untreated samples. For all groups, $n = 6$.

Periodate treatment of L_4 ES antigens prior to antibody probing revealed a significant reduction (p=0.003) in the level of mucus IgA binding in previously infected sheep (Figure 2.9, IgA graph).

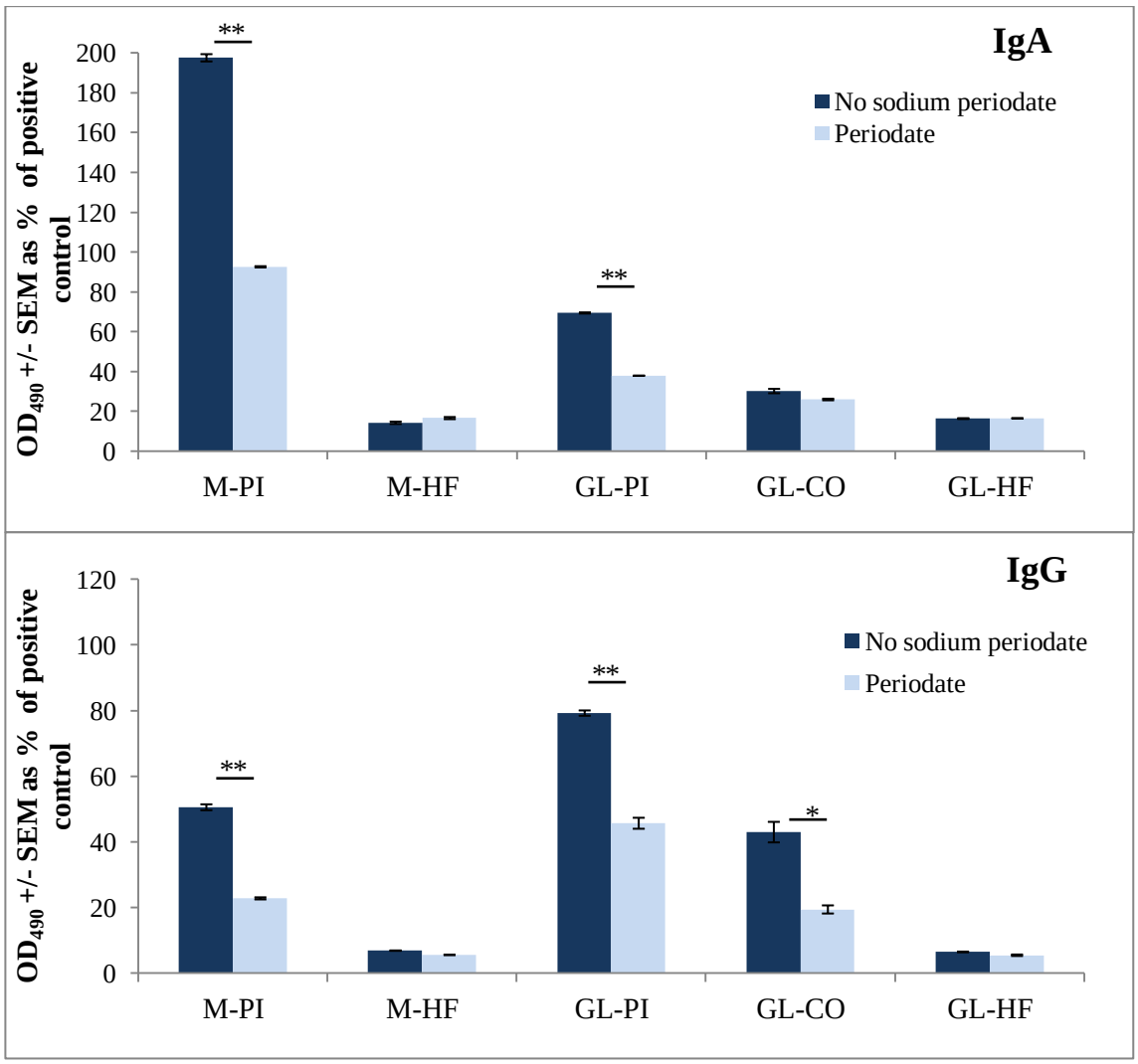

Figure 2.9 Effect of sodium periodate treatment of L_4 ES products on IgA and IgG antibody binding to L_4 ES antigens.

The mean OD (± SEM) results are expressed as percentage of the positive control. The positive control was efferent gastric lymph pooled from previously infected/challenged sheep over 6-10 dpc. Group "M-PI", abomasal mucus pooled from previously infected sheep. Group "GL-PI", efferent gastric lymph from previously infected sheep. Group "GL-CO", efferent gastric lymph from sheep given a single infection. In all groups the sheep were given a single bolus challenge of 50,000 *T. circumcincta* L_3. Group "M-HF", abomasal mucus pooled from helminth-naïve sheep. In the mucus groups, sheep were necropsied at 2 dpc to collect abomasal mucus. The gastric lymph samples were collected at 8 dpc. '**' and '*' denotes statistical significance ($p<0.01$, $p<0.05$) following comparison of the mean OD values between individual treated and untreated samples. For all groups, $n = 6$.

This reduction in antibody binding was observed in all the individual gastric lymph samples from the previously infected sheep. Similar results were seen in the level of IgG binding in gastric lymph from previously infected sheep following periodate treatment of L_4 ES antigens ($p=0.004$). However, in the IgG ELISA, periodate treatment of L_4 ES antigens also significantly reduced ($p=0.02$) the level of IgG binding to the antigens in samples from sheep given a single infection (Figure 2.9, IgG graph).

2.3.3 Immunoreactivity of IgA and IgG in mucosal washings against *T. circumcincta* antigens

Coomassie stained protein profiles of L_3, L_4, adult somatic extracts and L_4 ES products were separated by electrophoresis (refer to Section 2.2.6) as shown in Figure 2.10.

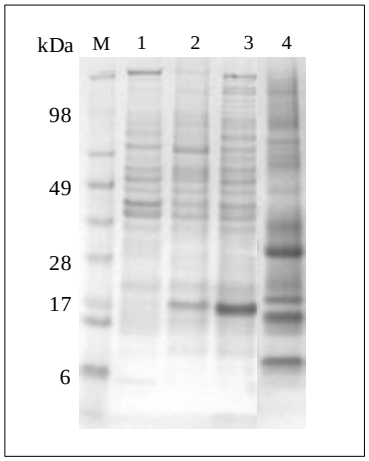

Figure 2.10 SDS-PAGE protein profiles of L_3, L_4 and adult *T. circumcincta* somatic extracts and L_4 excretory/secretory (ES) products.
Somatic extracts were separated by electrophoresis under reducing conditions on a 4-12% Bis-Tris NuPAGE gel and peptides visualised by Simply Blue stain. For each track, 10µg of somatic extract or ES products were loaded. Lane M: Molecular weight markers. Lane 1: L_3 somatic extract. Lane 2: L_4 somatic extract. Lane 3: Adult somatic extract. Lane 4: L_4 ES products.

In the L_3 somatic extract (Figure 2.10, Lane 1), around 15 distinct bands, ranging from 20 to over 100 kDa were present. In lanes containing somatic extracts from L_3, L_4 and adult (Figure 2.10, Lanes 1, 2 and 3 respectively), there were similarities in the banding patterns in the regions of 10-24 kDa and 40-80 kDa. Comparison of the banding pattern of L_4 somatic extract (Lane 2) with that of L_4 ES products (Lane 4) shows that during

the first 24 h of *in vitro* maintenance a wide range of proteins are produced, of which some of which may be ES-specific.

Immunoblots of somatic extracts prepared from L_3, L_4 and adult stages of the parasite were incubated with abomasal mucus from animals that were subjected to a trickle infection/bolus challenge of *T. circumcincta* L_3 (Figures 2.11 and 2.12). Binding of abomasal IgA antibody to antigens in all parasite stages examined was evident (Figure 2.11). The profile of antibody binding to L_3 antigens showed immunoreactive bands at 30 kDa, and 50–100 kDa. In the L_4 and adult somatic extracts, the profiles of abomasal IgA binding were similar and were against higher molecular weight antigens (90–200 kDa). Somatic extracts probed with pooled antibody from sheep given a single infection were not reactive to IgA (Figure 2.11, Lanes 4-6).

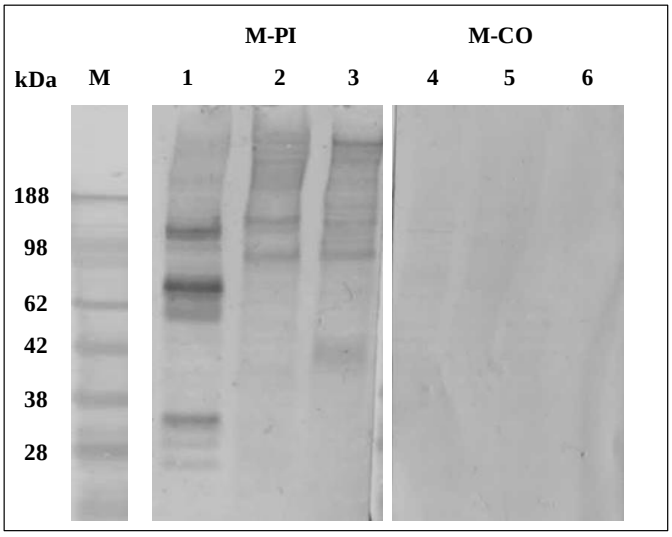

Figure 2.11 Immunoblot of *T. circumcincta* L_3, L_4 and adult somatic extracts probed for reactivity to mucus IgA from previously infected and primary infected sheep.

"M-PI" represents abomasal mucus from previously infected sheep (n=4). "M-CO" represents abomasal mucus from sheep given a single infection (n=4). In both groups sheep were given a single bolus challenge of 50,000 *T. circumcincta* L_3 and necropsied two days later to collect abomasal mucus. Lanes 1 and 4, L_3 somatic extract. Lanes 2 and 5, L_4 somatic extract. Lanes 3 and 6, Adult somatic extract. Lane M, molecular weight marker shown in kDa. Following incubation in the samples of abomasal mucus, all lanes were incubated with monoclonal mouse anti-bovine/ovine IgA, then polyclonal anti-mouse immunoglobulins-HRP. Strips were developed with SigmaFast™ DAB.

The pattern of IgG binding to somatic extracts of L_3, L_4 and adult parasites was investigated by western blotting (Figure 2.12). IgG reactivity was detected in all extracts and there were differences in the staining patterns between the three somatic extract preparations. Numerous IgG-reactive bands were detected across the range of 30–200 kDa in the L_3 somatic extract (Figure 2.12, Lane 1). Abomasal mucus from trickle-infected/bolus-challenged sheep bound to L_4 somatic antigens across the range of 20–200 kDa, with strong reactivity at approximately 45 kDa and 50 kDa (highlighted by arrows in Figure 2.12, Lane 2). Antigens (40–190 kDa) present in the adult somatic extract were also bound by IgG in the pool of abomasal mucus from trickle-infected/bolus-challenged sheep. Somatic extracts probed with mucus antibody from sheep given a single challenge did not show IgG reactivity (Figure 2.12, Lanes 4-6).

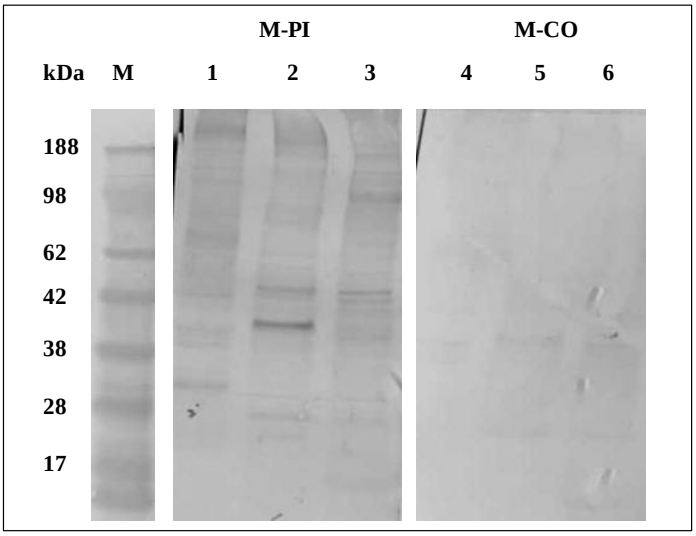

Figure 2.12 Immunoblot of *T. circumcincta* L_3, L_4 and adult somatic extracts probed for reactivity to mucus IgG from previously infected and primary infected sheep.

"M-PI" represents abomasal mucus from previously infected sheep (n=4). "M-CO" represents abomasal mucus from sheep given a single infection (n=4). In both groups the sheep were given a single bolus challenge of 50,000 *T. circumcincta* L_3 and necropsied two days later to collect abomasal mucus. Lanes 1 and 4, L_3 somatic extract. Lanes 2 and 5, L_4 somatic extract. Lanes 3 and 6, Adult somatic extract. Lane M, molecular weight marker shown in kDa. Following incubation in the pooled samples of abomasal mucus, all lanes were incubated with monoclonal mouse anti-ovine IgG-HRP. Strips were developed with SigmaFast™ DAB. Blue arrows highlight strong IgG-reactivity to antigens present at approximately 45 kDa and 50 kDa.

IgA in abomasal mucus from previously infected/challenged sheep bound to antigens in L_4 ES products across the range 14-100 kDa (Figure 2.13, Lane 1), which was not observed in the lane probed with abomasal mucus from sheep given a single infection (Figure 2.13, Lane 2). In the lanes probed for IgG binding, individual immunoreactive bands at approximately 10-14 kDa, 28 kDa, and 45-65 kDa were detected in the lane probed with abomasal mucus from previously infected/challenged sheep (Figure 2.13, Lane 3).

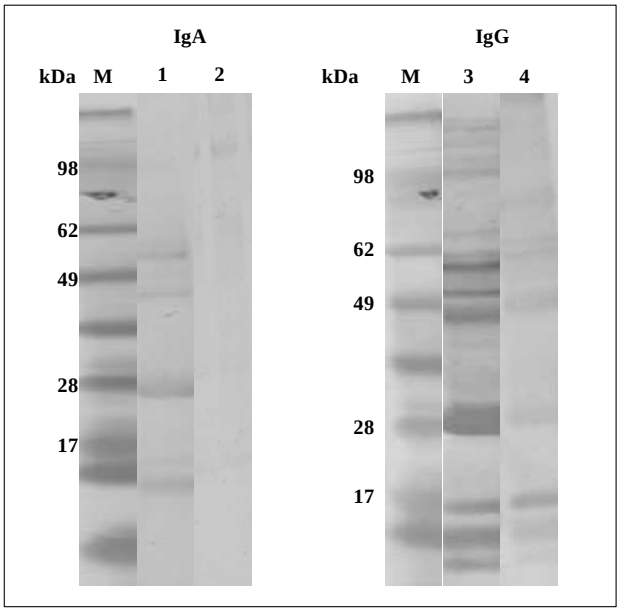

Figure 2.13 Immunoblots of *T. circumcincta* L_4 ES products showing abomasal mucus IgA and IgG binding to L_4 ES antigens.

ES (5µg) products from L_4 were subjected to electrophoresis under reducing conditions and transferred to a nitrocellulose membrane. Lanes 1 and 3 were incubated with abomasal mucus from trickle-infected/bolus-challenged sheep (n=4). Lanes 2 and 4 were incubated with a pool of abomasal mucus from sheep given a single infection (n=4). All sheep had been necropsied two days post-bolus challenge to collect abomasal mucus. Following incubation in abomasal mucus, all lanes were incubated as in Fig 2.11 for IgA and Fig 2.12 for IgG reactivity. Lane M, standard molecular weight markers given in kDa.

Mucus IgG activity against L_4 ES antigens was detected in abomasal mucus from sheep given a single infection (Figure 2.13, Lane 4). This pattern of immunoreactivity had a similar profile to the lane probed with mucus from previously infected sheep; however, the staining was not as intense (Figure 2.13, Lane 4).

2.3.4 The location of glycan moieties on immunoreactive antigens

Work presented in this chapter so far has highlighted that carbohydrate moieties on *T. circumcincta* larval antigens contribute to the level of antibody binding in abomasal mucus and gastric lymph (Section 2.3.2). For further investigation of these carbohydrate moieties, immunoblots of larval antigens were treated with sodium periodate prior to antibody probing. These immunoblots were compared to immunoblots not treated with sodium periodate to allow visualization of the effect of sodium periodate treatment on parasite antigens.

The optimal concentration of sodium periodate used to treat larval antigens immobilised on membranes was determined by staining for proteins following sodium periodate treatment. The protein profiles of L_3 somatic extract following treatment with a concentration range of sodium periodate are shown in Figure 2.14.

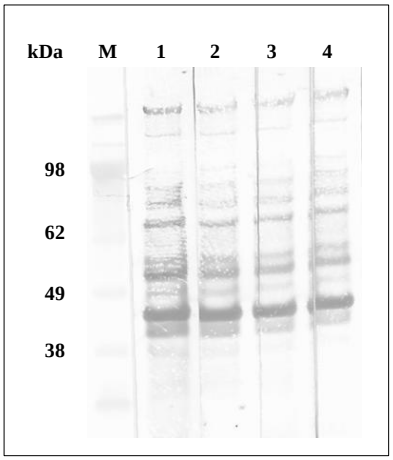

Figure 2.14 Detection of proteins in *T. circumcincta* L_3 somatic extract following sodium periodate treatment of antigens fixed on nitrocellulose membrane.

L_3 somatic extracts (5µg) were subjected to electrophoresis under reducing conditions, transferred to nitrocellulose membrane and strips thereof incubated with a concentration range of sodium periodate. Lane 1, no sodium periodate. Lane 2, 10mM sodium periodate. Lane 3, 20mM sodium periodate. Lane 4, 50mM sodium periodate. Protein was detected by Simply Blue stain (Invitrogen). Lane M denotes molecular weight standards which are marked in kDa.

The first track contains L_3 somatic extract (5µg) not exposed to sodium periodate (0mM) and was used as a reference lane for comparison with the treated extracts. All concentrations tested (10-50mM) showed a similar profile to the untreated sample. The optimal concentration for the sodium periodate treatment of immunoblots to disrupt carbohydrate moieties, but leave proteins intact, prior to antibody probing was determined to be 50mM.

Treatment of immunoblots of L_3 somatic extract with sodium periodate prior to probing with antibodies in abomasal mucus from previously infected sheep resulted in depletion of immunoreactive IgA bands observed in the range 50-200 kDa (Figure 2.15, IgA, Lanes 2 and 4). In contrast, the IgA-reactivity of bands detected in the L_3 extract at approximately 20-30 kDa (Figure 2.15, IgA, Lanes 2 and 4) were not affected by sodium periodate treatment. Sodium periodate did not have an obvious effect on IgG reactivity to *T. cirumcincta* L_3 somatic extract as there was no visual differences between sodium periodate-treated and untreated lanes following probing with mucus from previously infected sheep (Figure 2.15, IgG, Lanes 2 and 4).

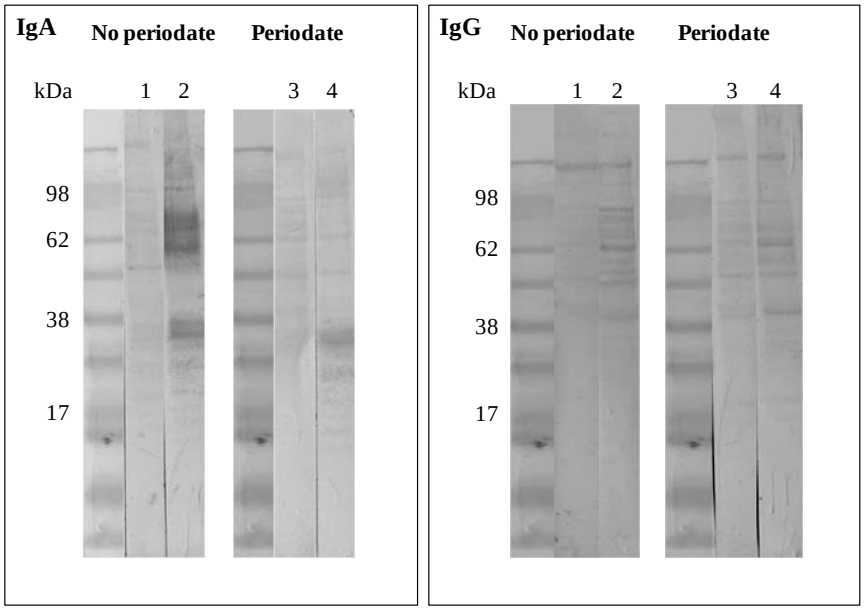

Figure 2.15 Immunoblots demonstrating the effect of sodium periodate treatment of *T. circumcincta* L_3 somatic extract upon the immunoreactivity of L_3 antigens to mucosal IgA and IgG.

Lanes 1 and 2, no sodium periodate. Lanes 3 and 4, antigens were treated with 50mM sodium periodate prior to antibody probing. Lanes 1 and 3, probed with pool of abomasal mucus from sheep given a single infection (M-CO). Lanes 2 and 4, probed with pool of abomasal mucus from given a trickle infection/bolus challenge (M-PI). All lanes were incubated with the appropriate secondary and tertiary antibodies for the isotype under investigation, and developed with SigmaFast DAB. Lane M represents molecular weight markers given in kDa.

Binding to L_4 somatic antigens of abomasal mucus IgA and IgG in sheep previously infected/challenged or given a single infection is shown in Figure 2.16. IgG immunoreactivity to the high molecular weight L_4 antigens (approximately above 90 kDa) (Figure 2.16, Lane 2) was depleted following preincubation of the blot with sodium periodate (Figure 2.16, Lane 4). IgA binding to a range of L_4 somatic antigens (20–150 kDa) was detected in abomasal mucus from previously infected sheep (Figure 2.16, Lane 2). Following periodate treatment, the IgA reactive bands across the range of 40–60 kDa were retained; however, reactivity at 28 kDa and 100 kDa was depleted (Figure 2.16, Lane 4). Mucus IgA and IgG activity against L_4 somatic antigens was not detected in sheep given a single infection (Figure 2.16, Lanes 1 and 3).

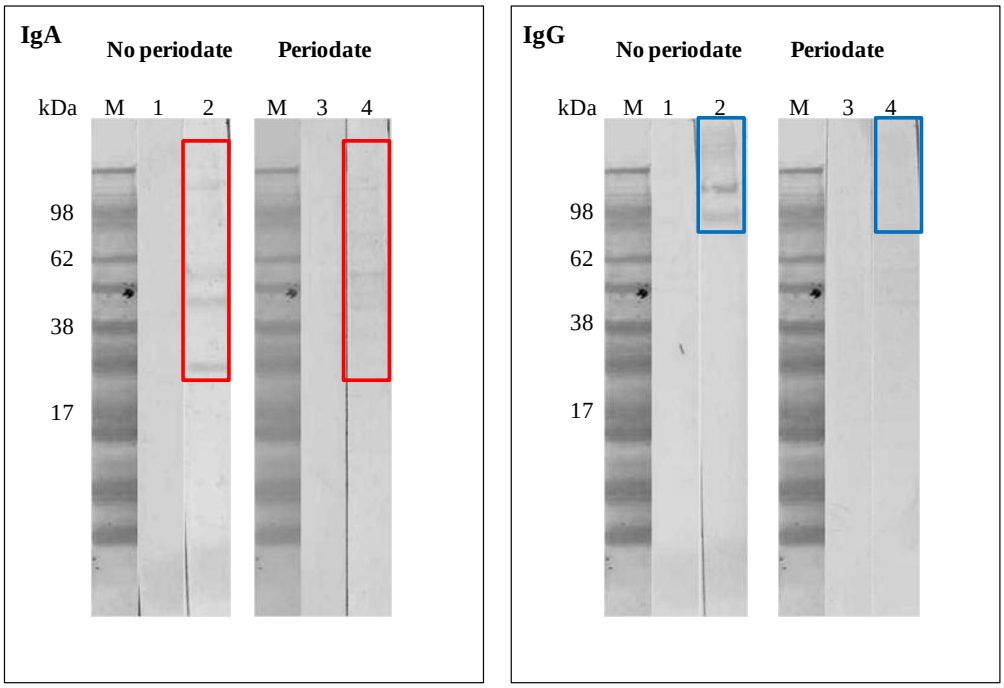

Figure 2.16 Immunoblots showing the effect of sodium periodate treatment of L4 somatic extract towards the immunoreactivity of L_4 antigens to mucus IgA and IgG.

Lanes 1 and 2, no sodium periodate. Lanes 3 and 4, treated with 50mM sodium periodate prior to antibody probing. Lanes 1 and 3, probed with pool of abomasal mucus from sheep given a single infection (M-CO). Lanes 2 and 4, probed with abomasal mucus from trickled-infected/bolus-challenged sheep (M-PI). After primary antibody incubations, all lanes were incubated with the appropriate secondary and tertiary antibodies for the isotype under investigation, and developed with SigmaFast DAB. Lane M, standard molecular weight markers given in kDa. Area of L_4-IgA reactivity affected by sodium periodate treatment highlighted by red boxes. Area of L_4-IgG reactivity affected by sodium periodate treatment highlighted by blue boxes.

Antigens from L_4 ES products were treated with sodium periodate prior to antibody probing for the detection of IgA and IgG binding (Figure 2.17). Mucus IgG binding to L_4 ES antigens (30-200 kDa) was detected in abomasal mucus from previously infected sheep (indicated in brackets on the blot), and following periodate treatment this immunoreactivity was reduced (Figure 2.17, Lane 3). The lanes probed with abomasal mucus from sheep given a single infection showed no IgA and IgG binding to L_4 ES antigens (Figure 2.17, Lanes 2 and 4). A large area of IgA reactivity was detected against L_4 ES antigens (range of 4–200 kDa) when probed with abomasal mucus from previously infected/challenged sheep (Figure 2.17, Lane 1). Following periodate

treatment, IgA immunoreactivity >90 kDa (highlighted on Figure 2.17, Lane 1) was reduced (Figure 2.17, Lane 3).

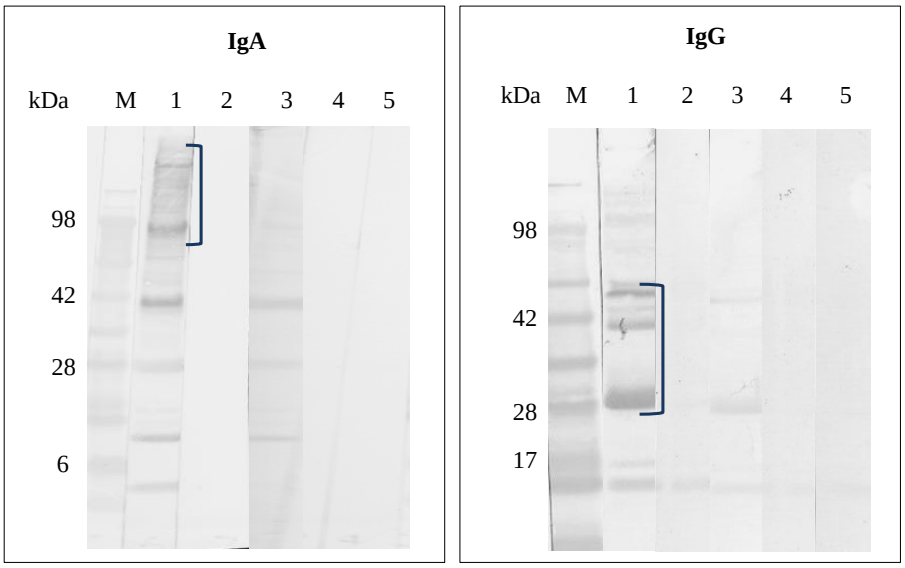

Figure 2.17 Immunoblot showing the the effect of sodium periodate treatment of L_4 ES antigens on mucus IgA and IgG binding to L_4 ES antigens.

Lanes 1, 2 and 5, no sodium periodate treatment. Lanes 3 and 4, treated with 50mM sodium periodate prior to antibody probing. Lanes 1 and 3 were incubated with pooled abomasal mucus from previously infected/bolus-challenged sheep (M-PI). Lanes 2 and 4 were incubated with pooled abomasal mucus from sheep infected with a single bolus infection of 50,000 L_3. Lanes 5, no primary antibody controls. Following incubation with the abomasal mucus samples, lanes were incubated with the correct combination of secondary and tertiary developing antibodies for the isotype under investigation, and developed with SigmaFast DAB™. Lane M, standard molecular weight markers given in kDa. Areas of IgA and IgG reactivity that were affected by treatment are highlighted by brackets.

The patterns of IgG reactivity detected in the lanes probed with gastric lymph from previously infected/challenged sheep were similar to each other as binding >28 kDa was detected in all lanes (Figure 2.18, Panel A, Lanes 3–5). IgG reactivity was affected by periodate treatment of L_3 somatic antigens; there was a reduction in the intensity of the binding detected (Figure 2.18, Panel B, Lanes 3–5).

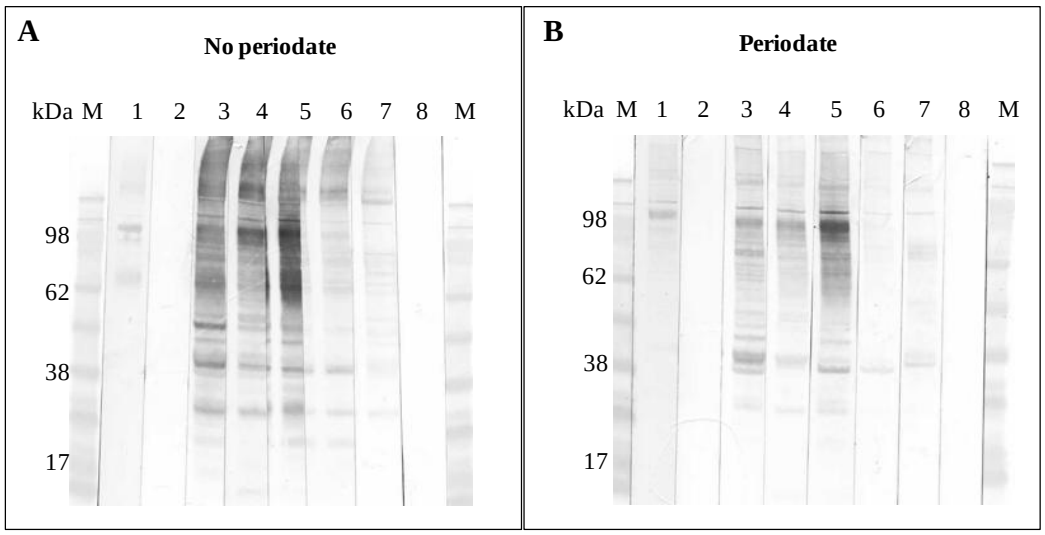

Figure 2.18 Immunoblots of *T. circumcincta* L_3 somatic antigens probed with efferent gastric lymph samples showing the effect of sodium periodate treatment of L_3 antigens on IgG binding.

Panel A, not treated with sodium periodate. Panel B, antigens treated with 50mM sodium periodate prior to antibody incubations. Lane 1, probed with abomasal mucus from previously infected/challenged sheep (*n*=4). Lane 2, probed with abomasal mucus from helminth-naive sheep (*n*=4). Lanes 3–5, probed with efferent gastric lymph from previously infected/challenged sheep. Lanes 6 and 7, probed with efferent gastric lymph from sheep given a single bolus infection. Lane 8, no primary antibody control. The gastric lymph samples were from a single time-point, 8dpc. Following primary antibody incubations, lanes were incubated with monoclonal mouse anti-ovine IgG-HRP and developed with SigmaFast DAB™. Lane M, molecular weight markers given in kDa.

IgG present in gastric lymph from animals subjected to a single infection (Figure 2.18, Lanes 6–7) bound to L_3 somatic antigens across the molecular weight range of 28 kDa and above; however, this binding was not as intensely stained as in the previously infected/challenged sheep. Binding of IgG to L_3 antigens was not evident in the no primary antibody control lane (Figure 2.18, Lane 8). Abomasal mucus from previously infected/challenged sheep contained IgG that bound to L_3 somatic antigens as indicated by reactive bands detected at 60–200 kDa (Figure 2.18, Panel A, Lane 1). This reactivity did not appear to be affected by sodium periodate treatment (Figure 2.18, Panel B, Lane 1). L_3 somatic antigens were not bound by IgG in the abomasal mucus of helminth-naive sheep (Figure 2.18, Lanes 2).

Areas of IgA reactivity against L_3 somatic antigens were detected when the extract was incubated with abomasal mucus and efferent gastric lymph from previously

infected/challenged sheep (Figure 2.19, Panel A, Lanes 1, 3, 4 and 5). Following sodium periodate treatment, IgA reactivity were affected as the intensity of binding was reduced (Figure 2.19, Panel B, Lanes 1, 3–5). The profiles of IgA reactivity against L_3 somatic antigens were very similar amongst all the gastric lymph samples from individual sheep (Figure 2.19). The lanes of L_3 somatic extract probed with gastric lymph from sheep exposed to a primary bolus infection did not demonstrate IgA binding (Figure 2.19, Lanes 6 and 7).

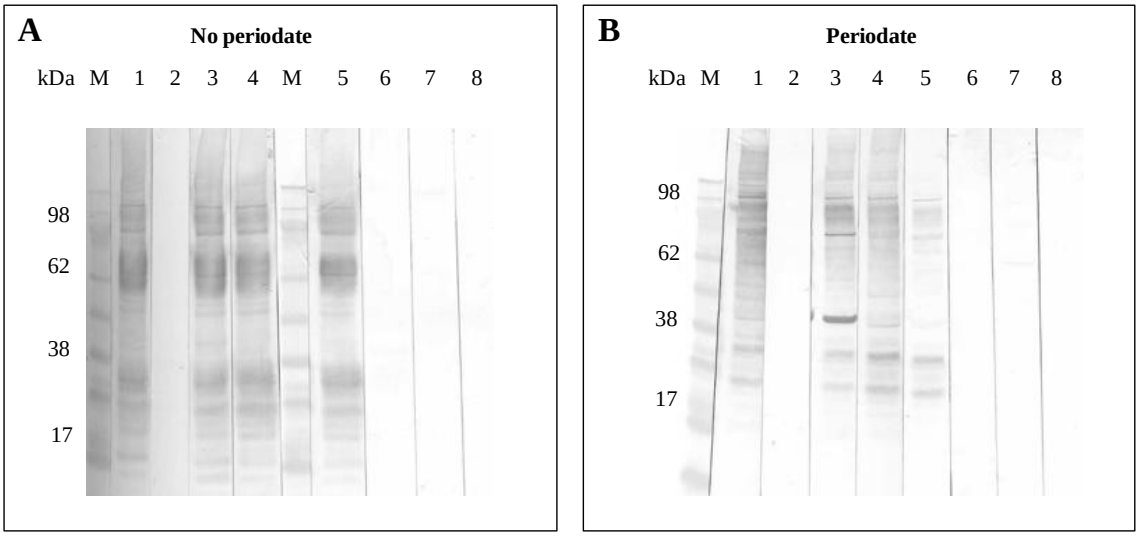

Figure 2.19 Immunoblots of L_3 somatic antigens probed with efferent gastric lymph samples showing the effect of sodium periodate treatment of L_3 antigens on IgA binding.

Panel A, not treated with sodium periodate. Panel B, antigens treated with 50mM sodium periodate prior to antibody incubations. Lane 1, probed with a abomasal mucus from previously infected/challenged sheep (*n*=4). Lane 2, probed with abomasal mucus from helminth-naive sheep (*n*=4). Lanes 3–5, probed with efferent gastric lymph from previously infected/challenged sheep. Lanes 6 and 7, probed with efferent gastric lymph from sheep given a single infection. Lane 8, no primary antibody control. The gastric lymph samples were from a single time-point, 8 dpc. Following primary antibody incubations, lanes were incubated with monoclonal mouse anti-bovine/ovine IgA, polyclonal anti-mouse immunoglobulins-HRP and developed with SigmaFast DAB™. Lane M, molecular weight markers given in kDa

2.3.5 Anamnestic response to L_3 somatic antigens

Antigen-specific ELISAs were used to investigate antibody responses to L_3 somatic antigens across a time frame in previously infected sheep. Periodate treatment of antigens was used to determine if carbohydrate moieties on larval antigens contributed to the immune response. Efferent gastric lymph samples from sheep subjected to an experimental trickle infection/challenge protocol were used to investigate antibody responses to L_3 somatic antigens before and up to 10 days after challenge with 50,000 *T. circumcincta* L_3 (Figure 2.20). For this part of the study efferent gastric lymph samples from two sheep which were classified as 'high-responders, as they had previously been demonstrated to have high total IgA concentrations in their efferent gastric lymph at 5-10 dpc (Halliday *et al*., 2007), were used. In both sets of gastric lymph samples, the level of IgA binding to L_3 antigens across -2 to 2 dpc remained at a similar level (Figure 2.20, IgA). At 3 dpc, the level of IgA binding began to increase, and in the samples from #A1008 IgA levels peaked at 6 dpc whereas in #A295 IgA levels increased until the final time-point of 10 dpc (Figure 2.20, IgA). Due to the rapidity of the IgA response, this suggests that an anamnestic IgA response to L_3 somatic antigens was evident. The L_3 specific-IgG levels in the efferent gastric lymph from both previously infected sheep showed little fluctuation (Figure 2.20, IgG).

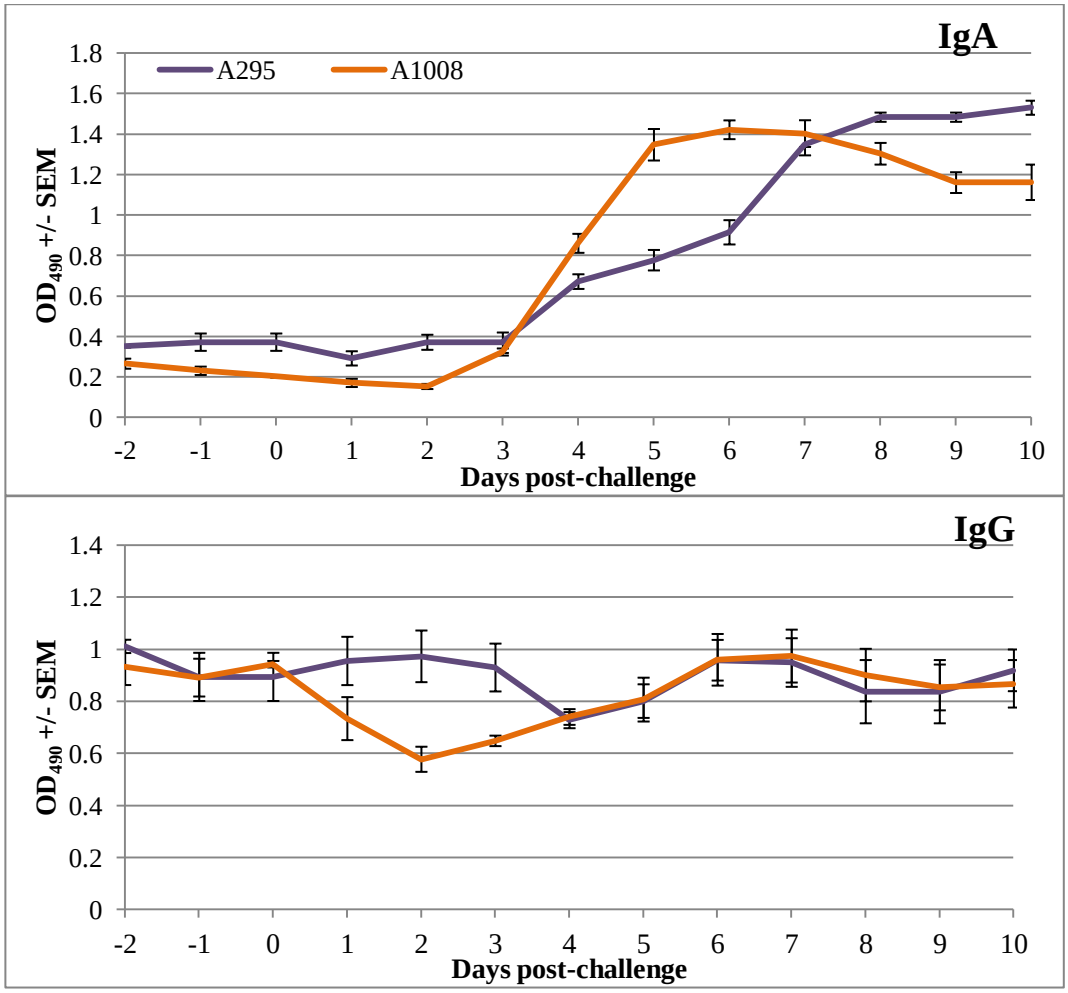

Figure 2.20 Gastric lymph IgA and IgG activity to *T. circumcincta* L_3 somatic antigens across the time period of -2 to 10 days post bolus L_3 challenge in two previously infected sheep.

Plates were coated with L_3 somatic antigen (5µg/ml) and probed with efferent gastric lymph samples (-2 dpc to 10 dpc) from two previously infected/challenged sheep. The two individual sheep were selected as they had been identified as 'high-responders' in a previous study. Orange line, #A1008. Purple line, #A295. Results are presented as mean OD ± SEM.

Periodate treatment of L_3 antigens prior to antibody probing was used to examine the contribution of carbohydrate moieties towards antibody binding across a time frame in previously infected sheep. Efferent gastric lymph samples which had previously been collected from two trickle-infected/challenged sheep across the time period of -2 to 10 dpc were used as the antibody probes. Due to the limited availability of gastric lymph from individual sheep across the time period of -2 to 10 dpc only two sheep were able to

be used which mean the power of any statistical test will be too weak to allow statistical analysis to indicate biological importance. In sheep #A295, periodate treatment of L_3 somatic antigens reduced the level of IgA binding at the following time-points: -2, -1, 1, 2 and 3 dpc (Figure 2.21, Panel A).

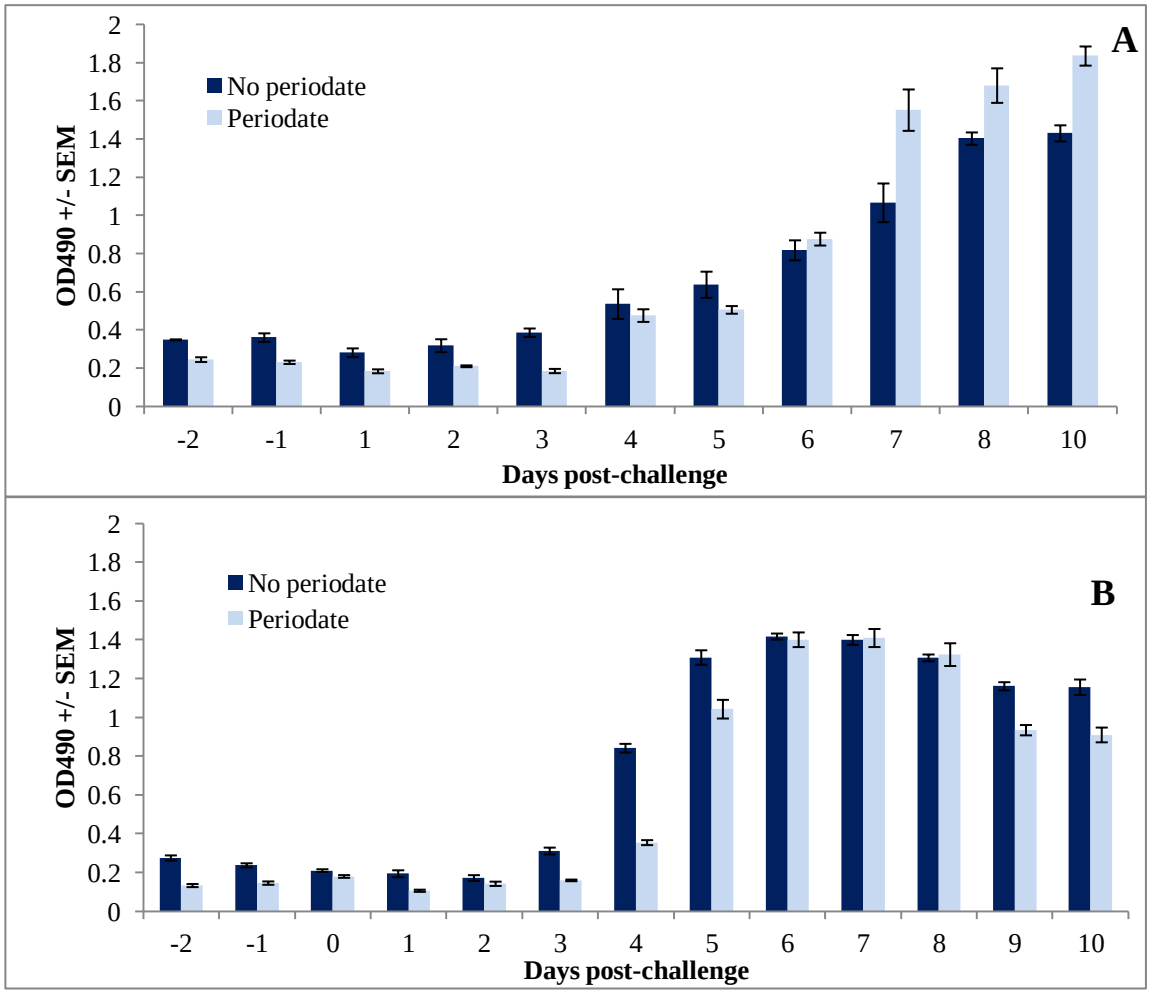

Figure 2.21 Effect of sodium periodate treatment of L_3 somatic extract on IgA antibody binding in gastric lymph samples from previously infected sheep.

Plates were coated with L_3 somatic antigen, and probed with gastric lymph from previously infected/challenged sheep. Samples were across the time period of -2 to 10 dpc. Panel A, #A295 and panel B, #A1008. Dark blue bars represent L_3 somatic extract not treated with periodate. Light blue bars represent L_3 somatic extract treated with periodate prior to antibody probing. Results are presented as mean OD ± standard error of six readings.

In contrast, periodate treatment of L_3 antigens prior to antibody probing was seen to increase the level of IgA binding at 7, 8 and 10 dpc. Using gastric lymph samples from sheep #A1008, the level of IgA binding to L_3 somatic antigens following periodate treatment of L_3 somatic extract was reduced at the following time-points: -2, -1, 0, 1, 3, 4, 5, 9 and 10 dpc (Figure 2.21, Panel B).

2.4 Discussion

In this chapter, interactions between antigens in extracts of various developmental stages of *T. circumcincta* and local antibody in previously infected/challenged and sheep given a single infection were investigated in detail. The antibody probes were generated from abomasal mucus and efferent gastric lymph, which had been collected in experimental trials in which sheep had been subjected to a *T. circumcincta* trickle infection and a single bolus challenge. The investigations demonstrated that IgA and IgG were reactive with a range of *T. circumcincta* antigens in larval somatic extracts and L_4 ES products. An anamnestic IgA response to L_3 antigens was evident in gastric lymph from trickle-infected/challenged sheep. Removal of carbohydrate moieties on the larval and ES antigens, by treatment with sodium periodate, significantly reduced the level of binding of IgA in gastric lymph and/or abomasal mucus from trickle-infected/challenged sheep. Further investigations revealed that a proportion of the local IgA reactivity was directed against glycans found on high molecular weight immunogenic antigens.

Numerous parasite antigens contain unique and immunogenic glycans, some of which may play a role in immune evasion (Hokke and Deelder, 2001; Nyame *et al*., 2003). Periodate treatment of immunoblots and ELISAs here indicates that carbohydrate moieties on antigens are involved in the reactivity of locally derived antibodies. Using abomasal mucus from trickle-infected sheep responding to a challenge infection as a source of antibody, it was demonstrated that periodate treatment of larval somatic extracts depleted IgA reactivity detected in the high molecular weight ranges 50-100 kDa and above 80 kDa in L_3 and L_4 blots, respectively. Carbohydrate moieties were also identified to be present in L_4 ES antigen preparations, as periodate treatment of L_4 ES depleted the high molecular weight IgA reactive regions (above 90 kDa). Carbohydrate epitopes and moieties have been found in a range of nematode parasite extracts, in particular larval and adult extracts from *H. contortus* (Schallig and Leeuwen, 1996; Van Stijn *et al*., 2010; Vervelde *et al*., 2003), adult ES products from *D. viviparus* (Kooyman *et al*., 2007) and L_3 extracts of *T. colubriformis* (Harrison *et al*., 2008). In a study investigating the presence of the LDNF glycan on the antigenic profile

of *H. contortus*, immunbolotting and a monoclonal antibody were used to identify glycoproteins in L_3 and adult extracts (Vervelde *et al*., 2003). The range of *H. contortus* L_3 antigens containing glycans were in the same molecular weight range to that reported here for *T. circumcincta* L_3. Earlier work in *H. contortus* revealed that sodium periodate treatment of antigens reduced the reactivity of larval and adult somatic extracts (Schallig and Leeuwen, 1996). Similar to the work presented here for IgA reactivity following periodate treatment of larval antigens, Schallig and Leeuwen (1996) found that treatment of L_3 somatic antigens with 20mM sodium periodate resulted in the removal of the high molecular weight regions of serum IgG reactivity. Together, these reductions in antibody binding to L_3 antigens following periodate treatment suggests that carbohydrate residues can play an important role in antibody binding.

Periodate treatment of antigen-specific ELISAs showed that, for all the extracts tested, there was a significant reduction in the level of IgA binding in efferent gastric lymph compared to untreated samples using material from sheep subjected to a trickle infection/challenge regime. A similar study using periodate treatment of antigens from larval and adult extracts of *H. contortus* indicated that removal of carbohydrate moieties led to a reduction in the level of binding of serum IgG_1 from *H. contortus*-hyperimmunised sheep (Schallig and Leeuwen, 1996). The same concentration of sodium periodate (20mM) was used to treat antigens before antibody probing in the antigen-specific ELISAs in both Schallig and Leeuwen (1996) and the work presented here. Following periodate treatment of antigens, the reductions in absorbance readings were of similar proportions, as in both studies the level of binding decreased by an OD reading of 0.1-0.2. However, in Schallig and Leuwen (1996) these findings were focused on the serum IgG response, whereas the work here reports local IgA response to *T. circumcincta* antigens. Glycoconjugates have been identified in L_3 and adult extracts of *H. contortus* by using lectin panels to specifically identify and characterize the glycans present (Schallig and Leeuwen, 1996). In particular, the study demonstrated through periodate treatment of antigen-specific ELISAs that a degree of the antibody response was directed against the carbohydrate epitopes on the *H. contortus* antigens

(Schallig and Leeuwen, 1996). A vaccination trial in *H. contortus* with adult ES products revealed that a proportion of the ES-specific IgA and IgG antibodies were directed against glycan epitopes and were associated with protection against a challenge infection (Vervelde *et al.*, 2003). In these experiments, vaccination with the ES glycoproteins resulted in an 89% reduction in parasite egg output and a 54% reduction in worm burden compared with the adjuvant control group (Vervelde *et al.*, 2003), highlighting the importance of glycans in immunity to nematodes.

Glycan moieties have been suggested to be important in the immunity to the bovine lungworm *D. viviparus* through their identification in both L_3 and adult ES products (Kooyman *et al.*, 2007). The latter study used a deglycosylation enzyme, PNGase F, to remove glycosylation sites on antigen preparations. Following this, there were reductions in IgA, IgG and IgE reactivity to L_3 and adult antigens in sera obtained from primary infected calves in response to challenge infection (Kooyman *et al.*, 2007). Further investigations revealed that whilst the N-glycan moieties on *D. viviparus* L_3 and adult ES products induced a strong antibody response, they failed to elicit a long-lasting antibody response to a challenge infection (Kooyman *et al.*, 2007). From the results presented here, it is unclear if glycan moieties on *T. circumcincta* antigens are involved in the maintenance of the local IgA response. The results from Kooyman *et al.* (2007) raise the possibility that glycan moieties may be implicated in a strong host immune response and, through their influence on the conformation of antigenic proteins, could be important for the maintenance of a protective response.

The results from immunoblots and ELISAs here suggest that carbohydrate moieties on *T. circumcincta* larval antigens may contribute to the protective immune response in previously infected sheep. This potential role of glycans in immunity is supported by studies on schistosomiasis, as serum IgA, IgG and IgM antibodies in *Schistosoma mansoni*-infected humans have been shown to be specifically reactive against carbohydrate N-glycan antigens, LDN (GalNAcβ1-4GlcNAc-R) and LDNF (GalNAcβ1-4(Fucα1-3)GlcNAc-R) (Nyame *et al.*, 2003; 2004). Antibodies specific to

the LDN glycan in *S. mansoni* have a protective role against infection through direct lysis of the tegument of schistosomula *in vitro* (Nyame *et al*., 2003).

Carbohydrate moieties in parasitic extracts potentially have the ability to modulate the type of immune response generated. Glycan structures in the parasitic nematode, *Brugia malayi*, were found to play a role in the induction of a Th2-mediated immune response, as periodate-sensitive antigens were directly involved in the induction of IL-4 in the first 72 h following infection, highlighting a role in T-cell priming (Tawill *et al*., 2004). Anti-glycan immune responses may be beneficial in different ways. First, they could be beneficial to the parasite by the action of blocking binding of more protective anti-protein antibodies (Eberl *et al*., 2001). These blocking properties of anti-glycan antibodies have been shown in *S. mansoni*, with the eosinophil-mediated killing of the schistosomula, the infective stage of the parasite, being blocked by antibodies specific to a carbohydrate egg-antigen (Dunne *et al*., 1987). Another theory is that they could act as a "decoy" for the host, by mimicking glycan structures present in the host to allow the parasite to evade the immune response (Van Die and Cummings, 2010). Glycan structures which are shared by both the parasite and hosts include the Lewis^x, LDN and LDNF structures (Van Die and Cummings, 2010). As discussed earlier in Section 2.1, the Lewis^x epitope and LDN-based glycans have been found in extracts of *H. contortus* (Van Stijn *et al*., 2010; Vervelde *et al*., 2003), and it is proposed that they may be involved in the modulation of the immune response. Many helminths have the capacity to shift the host immune response to a $CD4^+$ Th2 cell response, which is proposed to allow the parasite to establish a chronic infection; glycans have been implicated in the priming of this type of response (Tawill *et al*., 2004; Thomas and Harn, 2004).

Studies have shown that an important effector mechanism in sheep infected with *T. circumcincta* are local antibody responses, which been linked to inhibition of the development of larvae (Halliday *et al*., 2007; Stear *et al*., 1995; Strain and Stear, 1999) and immediate hypersentivity responses linked to the regulation of worm burdens (Stear *et al*., 1995). Results here demonstrate that immunoblots of L_3 antigens probed with

abomasal mucus from trickle-infected/challenged sheep identified regions of IgA reactivity around 30 kDa and 50-100 kDa, and IgG reactivity in the range 30-200 kDa. The profile of immunoreactivity detected against L_3 antigens was similar to that identified previously when L_3 antigen preparations were probed with antibody-secreting probes derived from abomasal lymph node cells (Balic *et al*., 2003). The sheep in the latter experiments had been subjected to a trickle infection of 3,000 *T. circumcincta* L_3 weekly for a period of 9 weeks, and then, after a period of 12 weeks, they were administered an infection of 50,000 L_3 (Balic *et al*., 2003). In the current study, abomasal mucus IgA and IgG from trickle-infected/bolus-challenged sheep bound a wide range of antigens in L_4 somatic extract and ES products. The profile of IgA reactivity in the abomasal mucus to L_4 somatic antigens was similar to that observed in efferent gastric lymph obtained from trickle-infected sheep at 8 days following a L_3 challenge infection (Halliday *et al*., 2007). The L_4 ES products used in this study were generated from an *in vitro* culture of *T. circumcincta* larvae collected from the abomasum at 7 dpi, and previous studies have looked at local IgA reactivity to L_4 ES products collected at 1, 3 and 5 dpi (Smith *et al*., 2009). The profiles of mucosal IgA reactivity to L_4 ES antigens in the work presented in this thesis and reported in Smith *et al*. (2009) are similar. More specifically, there are IgA-reactive bands at approximately 26 kDa, 46 kDa and in the range >80 kDa in the 7 dpi ES products presented here and the 5 dpc ES products in Smith *et al*. (2009). Using efferent gastric lymph from two individual trickle-infected/bolus-challenged sheep as local antibody probes, there was a clear anamnestic IgA response to nematode antigens after the administration of the L_3 challenge in trickle-infected sheep. Previous studies have shown that a local anamnestic IgA response to L_4 antigens was evident as early as 5 dpc in gastric lymph obtained from trickle-infected/bolus-challenged sheep (Halliday *et al*., 2007; Smith *et al*., 1983; Smith *et al*., 1984). The results presented here using the same local antibody probes indicated that L_3 antigen-specific IgA levels began to increase at an earlier time-point of 4 dpc. However, there are limitations to this result as gastric lymph samples across the time frame of -2 to 10 dpc were only available from two sheep from a trickle-infected/bolus-challenged group.

Potential vaccine candidates have been found through targeting extracts of L_3. A carbohydrate larval antigen (CarLA) is an L_3-specific surface antigen, which is present in a number of strongylid gastrointestinal nematodes and antibodies generated against it have been associated with a reduction in larval establishment (Harrison *et al*., 2003a; 2003b; 2008). Here, using abomasal mucus and efferent gastric lymph obtained from sheep subjected to a trickle infection, bolus challenge protocol, an IgA-reactive L_3 antigen of a similar size to CarLA, approximately 34-38 kDa (Figure 2.11, Lane 1; Figure 2.19, Lanes 1, 3-5) was detected. Previously CarLA has been identified in *T. circumcincta* L_3 antigens (Harrison *et al*., 2003a; 2003b). This was discovered by probing an SDS-extract of *T. circumcincta* L_3 with intestinal mucus from sheep subjected to a trickle infection of *T. colubriformis* (infected with 40,000 L_3 on three separate occasions over 9-12 weeks) (Harrison *et al*., 2003a; 2003b). The exact nature of the L_3-specific CarLA antigen is yet to be elucidated. However, biochemical analysis has revealed that it contains sugars, amino sugars and fatty acids (Maass *et al*., 2009). Treatment of CarLA with proteinase K, which would degrade proteins if present, did not affect the immunoreactivity of the antigen and in turn suggests that CarLA has a carbohydrate moiety (Harrison *et al*., 2003b). Work presented in this chapter revealed that following sodium periodate treatment of *T. circumcincta* L_3 somatic antigens there was a slight reduction in the L_3-IgA reactive band (approximately 34-38 kDa) detected in abomasal mucus (Figure 2.19, Panel A, Lane 1) and gastric lymph (Figure 2.19 Panel A, Lanes 3-5) from sheep subjected to a trickle infection, bolus challenge protocol. This finding specifically supports the work from Harrison *et al*. (2003b) and Maas *et al*. (2009), in which CarLA is shown to have a carbohydrate component, which is involved in antibody binding and epitope structure. Antibodies specific to CarLA could be implicated in the protective mucosal antibody responses in sheep to trichostrongyloid infections (Harrison *et al*., 2003a; 2008; Maass *et al*., 2007). However, a vaccination trial with purified CarLA, indicated that it did not induce a humoral response in the mucus and serum of sheep infected with *T. colubriformis* (personal communication). Research into how CarLA interacts with monocyte-derived dendritic cells (DCs), a type of antigen processing cell, concluded that both the purified and native forms of CarLA

are recognised and internalized by DCs (Pernthaner *et al*., 2012). As a result of the interaction, the DCs were not able to mature but pro-inflammatory cytokines, IL-6 and TNF-α, were released (Pernthaner *et al*., 2012). Collectively, these results highlight the importance of the L_3-surface specific antigen CarLA in the immune response to trichostrongylid nematodes.

Local antibody probes derived from sheep trickle-infected and bolus-challenged with *T. circumcincta* L_3 were used in this study to look at isotype binding to larval antigens. This was investigated by two routes: (1) antigen-specific ELISAs and (2) immunoblots. Results from the two immunoassays were similar in terms of pattern of immunoreactivity. Abomasal mucus and gastric lymph IgA and IgG gave the highest level and intensity of binding to all larval antigens when probed with antibody probes from trickle-infected/challenged sheep. The probes derived from sheep receiving a single challenge infection and the helminth-naïve sheep showed little reactivity in both assays. The immunoblots of L_3 somatic extracts probed with the different antibody probes, abomasal mucus and gastric lymph showed differences in the level of binding. In particular, the IgG blots probed with efferent gastric lymph from sheep subjected to a trickle infection/challenge protocol showed stronger profiles of immunoreactivity compared to the blots probed with abomasal mucus. There were no clear differences between the two trials in terms of their experimental trickle infection and bolus challenge protocol. In the two separate experimental trials, the same dose of larvae was administered to the sheep over the same number of weeks and the sheep were killed at the same time-point of 2 days following the bolus challenge infection (Halliday *et al*., 2007; Knight *et al*., 2011). One possible explanation for the IgG reactivity demonstrated in the gastric lymph from the sheep subjected to a single bolus infection is that the cannulation method used to collect the efferent lymph from the common gastric lymph duct could have induced a local IgG response.

Abomasal mucus IgA from sheep subjected to a trickle infection have been used to select L_4 antigens that are targets of early immune response in trickle-infected sheep responding to a bolus challenge infection (Nisbet *et al*., 2010a). Proteomic approaches

were used to characterise components of L_4 ES products and led to the identification of some of the most abundant proteins, such as cathepsin F-1 (Tci-CF-1) (Redmond *et al*., 2006; Smith *et al*., 2009). Tci-CF-1 is a potential protective candidate as it may have a role in the development of parasites and is a target of local IgA response in trickle-infected sheep responding to a challenge infection (Redmond *et al*., 2006). Another immunogenic molecule, Tci-ASP-1, found in L_4 ES products is a member of a group of nematode-specific molecules referred to as 'activation-associated secreted proteins' (Nisbet *et al*., 2010a). These molecules are thought to play a key role in the establishment of parasites in their hosts (Hawdon *et al*., 1996; 1999) and could be protective against challenge infection. Work in subsequent chapters will focus on the identification of immunoreactive proteins from a somatic extract of *T. circumcincta* L_3 following an immunoaffinity purification process with local IgA from trickle-infected sheep in response to a challenge infection to select IgA-reactive antigens.

In this Chapter, the immunoreactivity investigations demonstrated that larval antigens from *T. circumcincta* are bound by local antibodies generated in response to a challenge infection in trickle-infected sheep. Further research revealed that glycan components are present in larval antigens and were bound by both IgA and IgG present in abomasal mucus and gastric lymph of trickle-infected/challenged sheep. Work in the next chapter will focus on the interactions of L_3 antigens with the host immune system through identification of the immunoreactive proteins.

Chapter 3 : Analysis of IgA-reactive *Teladorsagia circumcincta* larval proteins

3.1 Introduction

One traditional approach to identifying potential vaccine candidate molecules for the control of helminth infections is to perform systematic fractionation of parasite extracts to target molecules that may induce protective immune responses against challenge (Emery and Wagland, 1991; Emery *et al*., 1993). As whole worm extracts are complex mixtures, several fractionation techniques have been employed to simplify the mixtures, or to select for a class (for example, cysteine proteases) of specific molecules. Lectin affinity chromatography has used been to select glycosylated antigens from crude worm extracts (Smith *et al*., 1994), and affinity chromatography with thiol-sepharose has been used to purify thiol-binding molecules (Geldhof *et al*., 2002; Redmond and Knox, 2004). Another approach has been to use host antibodies to target protective antigens in parasite extracts or their excretory/secretory (ES) material (Murphy *et al*., 2010; Nisbet *et al*., 2009; 2010a; Smith *et al.*, 2009). These methods often include protein denaturation or reduction steps in the sample preparation protocols, which may result in the loss or change of structural epitopes required for appropriate antibody binding (Pathak and Palan, 2005).

Studies with *Haemonchus contortus* have revealed the importance of discontinuous or structural epitopes in immunity against nematodes (Munn *et al*., 1997; Smith and Smith 1996; Smith *et al.*, 2000). One prime example is with a microsomal aminopeptidase, termed H11, which was isolated from the interstinal microvilli of adult *H. contortus* (Smith and Smith, 1996). In a vaccination trial, the levels of protection afforded by vaccination of sheep with two different forms of the antigen, (i) native and (ii) denatured by using sodium dodecyl sulphate (SDS) and dithiothreitol, were compared (Munn *et al*., 1997). Following a challenge infection of 10,000 L_3 there was a reduction in the levels of protection (reductions of 21% and 41% in faecal egg counts and adult nematode burdens, respectively) afforded by the H11 antigen when denatured

compared to the untreated antigen (Munn *et al*., 1997). This demonstrates that structural epitopes were important for antibody binding and the protective immune response induced by vaccination with the native nematode antigen. Further evidence for the importance of structural epitopes is provided by research into a lead vaccine candidate for *H. contortus*, termed *Haemonchus* galactose-containing glycoprotein complex (H-gal-GP) (Smith *et al*., 2000). Vaccination with native H-gal-GP antigens induced 90% and 70% reductions in faecal egg output and adult nematode burdens, respectively, following a challenge infection of yearling sheep with *H. contortus* $L_{3,}$ compared to unvaccinated sheep (Smith *et al*., 1994; 2000). To identify components of the complex, native H-gal-G was dissociated and fractionated by gel filtration chromatography, revealing a high molecular weight fraction that contained four metalloendopeptidases (MEPs) (Smith *et al*., 2003a) and a low molecular weight fraction that contained pepsin-like aspartyl proteases (Smith *et al*., 2003b). The MEPs were fractionated further by non-reducing SDS-PAGE and vaccination with MEP-3 alone, or MEP-1, 2 and 4 in combination, resulted in a 33% mean reduction in faecal egg output after challenge, in comparison to challenged control sheep (Smith *et al*., 2003a). These vaccination studies with the H-gal-GP complex emphasise that epitopes present on the native antigen can be discontinuous, *i.e*. on separate sections of the antigen complex. In turn, these could be essential for antibody binding as fractionation/denaturation of the antigen complex was associated with a reduction in the antibody-based immunity afforded by vaccination.

A further approach used for identifying nematode vaccine candidates is based on the immunoscreening of protein extracts separated by SDS-PAGE (Schallig *et al*., 1997) or cDNA expression libraries (DeMaere *et al*., 2002; Newlands *et al*., 1999; Vercauteren *et al*., 2003) using polyclonal sera or local antibody probes derived from immune animals. The vectors used for cDNA library preparation are often bacterial (Dale and Von Schantz, 2007) and proteins expressed by these systems do not facilitate the correct post-translational modifications (for example, glycosylation (Gelhof *et al*., 2007; Nyame *et al*., 2004)) which are critical. This suggests that immunoscreening of bacterial cDNA libraries for protective antigens may miss glycosylated and other three dimensional

antigens due to differences in the linear structure compared to the native structure and components. Bacterial expression systems often express the antigen as insoluble inactive intracellular inclusion bodies, which can be associated with the absence of structural epitopes (Dalton *et al*., 2003).

The ability to identify protective antigens from complex organisms therefore requires a strategy that allows binding of antibodies to nematode components/molecules in their native conformation. In this chapter, an immunoaffinity chromatography method was used to target antigens separated under native conditions, in an attempt to target structural epitopes. Here, chromatography was used in combination with antibody binding experiments to target antigens/epitopes present in the L_3 stage of *T. circumcincta*. A similar strategy had been used previously in the development of a novel vaccine against the tick, *Rhipicephalus (Boophilus) microplus* (Opdebeeck *et al*., 1989): purified serum IgG derived from an animal immunised with tick mid-gut extract (Opdebeeck *et al*., 1988) was used as an affinity ligand to purify sub-fractions, which were then administered to cattle (Opdebeeck *et al*., 1989). Immunisation with these native antigen fractions elicited significant protection against challenge infection, with 80% reduction in the weight of eggs laid by ticks in immunised animals compared to non-immunised controls (Opdebeeck *et al*., 1989). Monoclonal antibodies, derived from mice immunised with gut microvilli surface antigens of *H. contortus*, were used to affinity-purify antigens from extracts of adult stages of this species (Jasmer *et al*., 1993). These affinity-purified antigens were shown to contain carbohydrate epitopes due to sensitivity to periodate oxidation (Jasmer *et al*., 1993). Subsequent immunisation of goats with antigens isolated through affinity for the monoclonal antibodies induced a 60% significant reduction in total worm counts following a *H. contortus* challenge infection in immunised goats compared with challenged control groups (Jasmer *et al*., 1993). To date, the immunoaffinity approach to purify antigens from extracts of *T. circumcincta* under native conditions, thereby retaining epitope structures, is an avenue which has not been explored in the development of a *T. circumcincta* vaccine.

Abomasal mucus IgA, which was generated from sheep administered a 50,000 L_3 challenge infection with *T. circumcincta* having previously been rendered immune by experimental trickle infection, has been positively correlated to the proportion of arrested larvae in the abomasum (Stear *et al.*, 1995; 1999). Using IgA from sheep experimentally-infected with a trickle infection of *T. circumcincta* L_3 over 8 weeks, vaccine candidate antigens have been identified in *T. circumcincta* L_4 ES products by immunoblotting with the abomasal mucus IgA (Nisbet *et al.*, 2010a; Redmond *et al.*, 2006). Here, the focus is the initial interaction between *T. circumcincta* and the sheep and so L_3 antigens were subjected to analysis because these stages directly encounter the host's response and physicochemical signals, which in turn can trigger processes to ensure the successful establishment of the parasite in its host niche (Kiel *et al.*, 2007). Nematode proteomes are, to some degree, differentially regulated at different points in the life cycle. The helminth proteome has been demonstrated to be dynamic in that the components present can change in response to specific alterations in environmental conditions and the immunological response by the host (Barrett, 2000; 2009). Here, the proteome of *T. circumcincta* at the initial point of contact and establishment in the abomasum was investigated to provide information on potential vaccine candidates.

The availability of helminth EST and genomic DNA databases, has led to the successful application of proteomics to target potential vaccine candidates (Wilson *et al.*, 2004; Smith *et al.*, 2009). Sequencing of the genome of *T. circumcincta* is still in progress (Accessed: February 2014, http://genome.wustl.edu/genomes/detail /teladorsagia-circumcincta/) and in February 2014 there were 6,061 *T. circumcincta* expressed sequence tags (ESTs) publically available (NCBI, http://www.ncbi.nlm.nih.gov). The identification of proteins contained in *T. circumcincta* L_4 ES products from parasites collected at 1, 3 and 5 dpi highlighted potential vaccine candidates; Tc-CF-1, astacin-like metalloprotease, activation-associated secreted (ASP) proteins and an aspartyl proteinase enzyme (Smith *et al.*, 2009). Tci-CF-1 is the most abundant component of L_4 ES and is a target of local IgA responses in sheep rendered immune to infection (Redmond *et al.*, 2006). Homologues

of ASP proteins were found in L_4 ES products and following recombinant expression of Tc-ASP-1 it was evident that the recombinant version is also bound by the IgA present in abomasal mucus from previously infected sheep (Nisbet *et al*., 2010a). Following these approaches, a recombinant cocktail vaccine against *T. circumcincta*, containing eight antigens identified by the various means listed above is currently under development and is showing promise as a viable vaccine (Nisbet *et al*., 2012). All of the previous work into vaccine development against *T. circumcincta* has focused on the identification of antigens present in L_4 worms and/or their ES products and there has been little focus towards the identification of the L_3 antigens which are targets of the ovine immune response.

Here, a multi-step immunoaffinity chromatography approach was used to identify IgA-reactive antigens present in somatic extracts of *T. circumcincta* L_3. A schematic illustration of the process is provided in Figure 3.1. Following isolation of the IgA-reactive L_3 fraction, liquid chromatography-electron spray ionisation-tandem mass spectrometry (LC-ESI-MS/MS; Ashton *et al*., 2001) was used to identify proteins eluted during the immunoaffinity purification step. The peptide data generated by mass spectrometry were then analysed to provide information on protein structure, peptide mass and amino acid sequence (Graves and Haystead, 2002) using the MASCOT (MatrixScience™) software, which also provided a molecular weight search (MOWSE) score for protein identification of each peptide (Pappin *et al*., 1993). The relative immunogenicity of the selected antigens was then assessed by measuring specific abomasal and gastric lymph IgA responses in experimentally infected sheep demonstrated, by parasite burden analysis, to have varying degrees of immunity. This was to confirm if components of purified L_3 fraction could act as potential vaccine candidates to target incoming *T. circumcincta* L_3 in infected sheep.

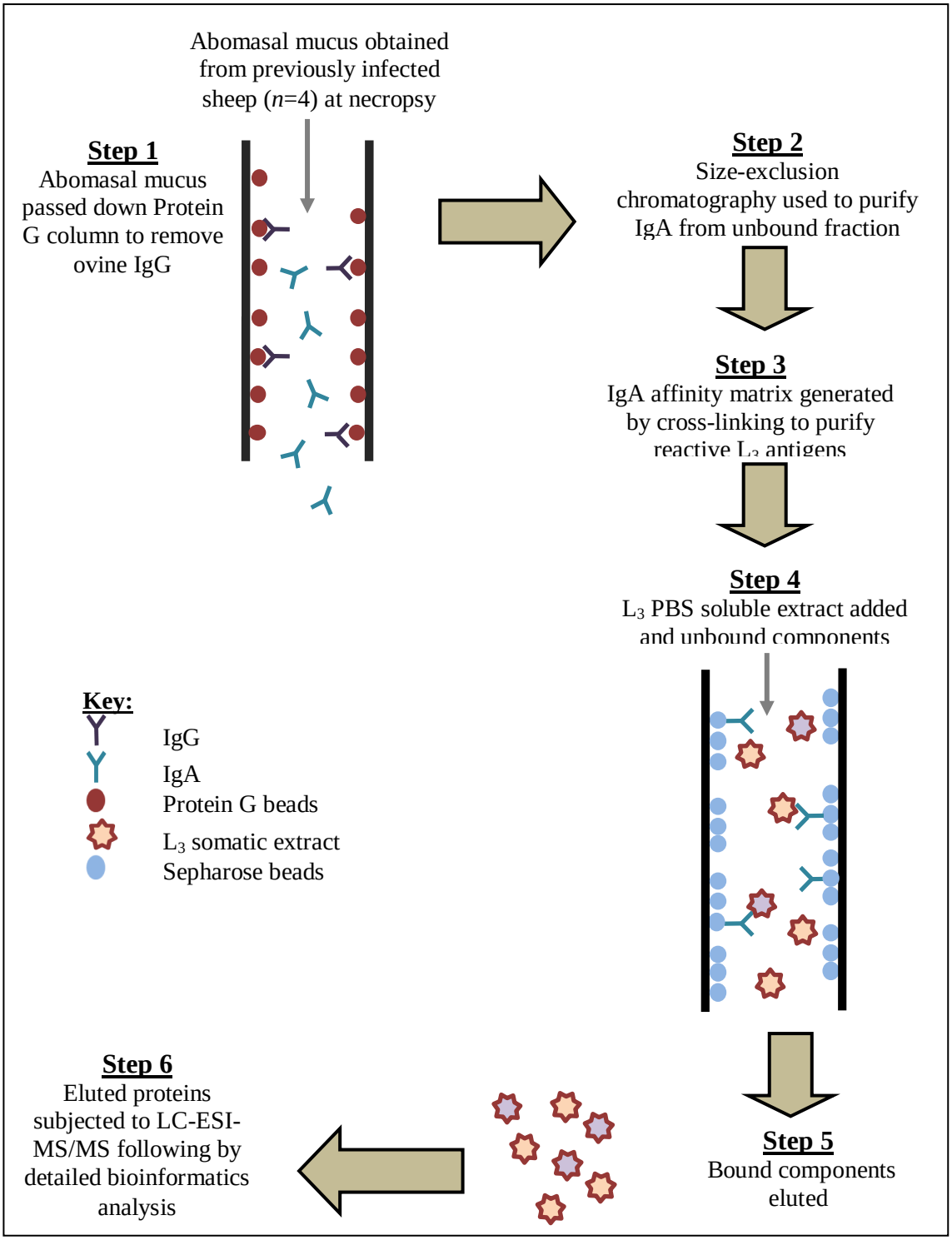

Figure 3.1 Schematic illustration of process for purification of IgA-reactive *T. circumcincta* L_3 antigens under native conditions.

3.2 Materials and Methods

3.2.1 Purification of IgA and IgG from abomasal mucus

A 5 ml pool of abomasal mucus obtained at post mortem from sheep previously infected then challenged with *T. circumcincta* L_3 (see section 2.2.1) was created, diluted 1:3 in PBS, pH 7.4, and centrifuged at 700 *g* for 10 min to remove insoluble material. The supernatant was loaded onto a 1 ml HiTrap Protein G column (GE Healthcare) pre-equilibrated with PBS. The column was attached to an FPLC chromatography machine (AKTA™ purifier UV-900/P-900) to allow monitoring of UV absorbance, pH, conductivity and sample collection time-points. The column was washed with 10 volumes of PBS and the unbound material collected (three x 10 ml fractions). Unbound protein, containing IgA and depleted of IgG, was concentrated to 1 ml at 4°C using Amicon centrifugal Ultra-4 devices with a 10 kDa molecular weight cut-off (MWCO) membrane. IgG bound to Protein G agarose beads on the column bed was eluted by the application of 2 ml of 0.5M NaCl in 0.1M citric acid, pH 2.5, and immediately neutralised with 150 µl of 1M Tris, pH 9.1, per ml of eluted fraction. The column was re-washed with 10 ml PBS to re-equilibrate for future use. Fractions containing purified IgG were aliquoted and stored at -20°C. The presence of IgA and IgG in the unbound and eluted fractions, respectively, was investigated by immunoblotting as described in Section 2.2.4, with the appropriate secondary and tertiary anti-IgA and -IgG antibodies. For detection of IgA, a mouse monoclonal anti-bovine/ovine IgA (Serotec, MCA628) diluted at 1:250 in TNTT was used as a secondary antibody and a polyclonal rabbit anti-mouse immunoglobulins-HRP (Dako, P0260), diluted at 1:1000 in TNTT, as a tertiary antibody. For detection of IgG, monoclonal mouse anti-goat/sheep IgG-HRP (Sigma, A0452), at 1:1000 in TNTT, was used as a secondary antibody.

3.2.2 Size-exclusion chromatography purification of IgA

To purify IgA in the unbound fraction further, the material was passed through a Superose 12 HR 10/30 column (GE Healthcare) coupled to FPLC apparatus at a flow rate of 0.3 ml/min. Prior to loading onto the column, the concentrated unbound fraction was centrifuged at 16000 *g* for 5 min to pellet any insoluble material, then 200 µl loaded onto the Superose 12 column (pre-equilibrated with PBS, pH 7.4) at a flow rate of 0.3 ml/min. The initial flow-through from the column was discarded (classified as 'void volume'; usually approximately 6ml). The chromatograph trace tracked the absorbance in UV units, and when UV units began to rise, the FPLC was programmed to collect 26 x 0.5 ml fractions, automatically. The run was repeated until the entire unbound fraction was depleted. Fractions containing IgA from across all the runs were pooled and stored at 4°C. To identify which fractions from the size-exclusion purification of the unbound fraction contained IgA, NuPAGE SDS gel analysis was performed (see Section 2.2.4) with the following modifications: 10 µl of sample were separated by electrophoresis in a final volume of 20 µl in each well. Two gels were run; one for protein staining, and the second for immunoblot detection of antibody isotypes IgA and IgG. For detection of IgA and IgG, immunoblotting steps were performed as in section 3.2.1.

3.2.3 Confirmation of immunoreactivity of purified IgA and IgG with *T. circumcincta* L_3 somatic extracts

Binding of purified IgA and IgG to antigens present in L_3 somatic extracts was investigated by immunoblotting. Briefly, 5 µg per lane of *T. circumcincta* L_3 somatic extract (prepared as described in section 2.2.4) were run on 4-12% NuPAGE SDS gels under reducing conditions (see section 2.2.6). Once run, one lane of the gel was stained using Simply Blue Stain and the remaining lanes were electro-blotted onto a 0.45 µmM nitrocellulose membrane (see section 2.2.7). To block non-specific binding sites, nitrocellulose membranes were incubated in TNTT overnight at 4°C. The appropriate

lanes were then incubated in primary antibody; either purified IgA or purified IgG (see Table 3.1) for 2 h at room temperature. Lanes were then washed in TNTT for 10 min, repeated 3 times. Secondary and tertiary antibodies were then applied (detailed in Table 3.1) for 1 h at room temperature, prior to 3 x 10 min TNTT washes. Following the final wash after incubation with the HRP conjugated antibody, detection was carried out using DAB (3', 3' diaminobenzidine, Sigma).

Primary antibody		**Secondary antibody**			**Tertiary antibody**		
	Dilution	**Description**	**Manufacturer**	**Dilution**	**Description**	**Manufacturer**	**Dilution**
IgA[1]	1 in 10	Mouse monoclonal anti-bovine/ovine IgA	Serotec, MCA628	1 in 250	Polyclonal rabbit anti-mouse Ig HRP-conjugated	Dako, P0260	1 in 1000
IgG[2]	1 in 10	Monoclonal mouse anti-goat/sheep IgG HRP-conjugated	Sigma, A0452	1 in 1000	N/A	N/A	N/A

Table 3.1 Antibodies used for the detection of ovine IgA and IgG on immunoblots

KEY:[1] IgA from previously infected/challenged abomasal mucus purified by Protein G affinity chromatography and size-exclusion chromatography. [2] IgG from previously infected/challenged abomasal mucus purified by Protein G affinity chromatography

3.2.4 Preparation of IgA custom NHS-sepharose immunoaffinity column

For creation of an IgA affinity column, pooled fractions containing IgA (see Section 3.2.2) were coupled to a HiTrap NHS-activated HP column, 1 ml capacity (GE Healthcare). The NHS-activated HP column was pre-equilibrated by the following steps. Isopropanol storage solution was washed out with 6 column volumes of 1 mM HCl, then 4 ml purified IgA, dissolved in coupling buffer (PBS, pH 7.4) at a concentration of 0.4 mg/ml (approximately 1.6 mg), were applied to the column through inlet tubing controlled by a peristaltic pump, and allowed to re-circulate for 45 min at room temperature at a flow rate of 0.5 ml/min. The column was sealed to allow the IgA to cross-link to the activated sepharose beads for 30 min at room temperature.

Deactivation of excess amine groups and washing of non-specifically bound protein was achieved through a series of buffer exchange washes with blocking buffer (0.5M ethanoloamine, 0.5M NaCl, pH 8.3) and wash buffer (0.1M sodium acetate, 0.5M NaCl, pH 4.0). The column was then re-equilibrated by washing with 12 column volumes of PBS, and stored at 4°C overnight. The coupling efficiency of protein ligand was measured by comparing the absorbance values, measured at 280nm (A280), of the IgA solution before and after coupling.

3.2.5 IgA immmunoaffinity chromatography purification of antigens from somatic extracts of *T. circumcincta* L_3

The IgA-NHS affinity column was equilibrated prior to immunoaffinity purification by washing with 10 column volumes of PBS at a flow rate of 0.5 ml/min. L_3 somatic extracts were centrifuged at 14000 *g* for 10 min to pellet any insoluble material. Approximately 10 ml supernatants (protein concentration 0.7mg/ml) were recirculated through the column at a flow rate of 5 ml/min for 16 h at 4°C. Following sample application, unbound material was washed from the column bed with 10 column volume washes of PBS at a flow rate of 0.5 ml/min, with the flow through retained. Bound antigens were eluted with 2 column volumes of 0.1M glycine-HCl, 6M urea, pH 2.5, and 10 x 1 ml fractions collected. The column was then re-equilibrated with 10 column volumes of PBS, and stored in 20% (v/v) ethanol. Eluted fractions (~10 ml) were pooled and concentrated to approximately 500 µl using Amicon Ultra-15 10kDa MWCO centrifugal devices at 3000 *g* for 20 min at 4°C. These were then buffer exchanged with PBS over three washes, and the remaining 500 µl transferred to Microcon YM-10 10 kDa NMWL centrifugal units. The concentrator units were centrifuged at 10000 *g* at 4°C for 20 min, until samples were concentrated to approximately 50 µl.

3.2.6 Proteomic analysis of IgA affinity-purified L_3 antigens

For proteomic analysis, 10 µl of concentrated affinity-purified L_3 extracts were fractionated by SDS-PAGE under reducing conditions. Ten µl sample were mixed with 5 µl SDS-PAGE sample buffer, 2 µl sample reducing agent (Invitrogen Lts, UK), boiled at 70°C for 10 min before loading onto 4-12% gradient gels. After electrophoresis, resolved proteins were visualised with colloidal Coomassie Blue (Simply Blue Safe Stain, Invitrogen), and destained in water. Gel tracks under investigation were sliced horizontally into 26 equal gel slices of 2.5 mm width. Each of the slices were chopped finely, transferred to clean 0.5 ml Eppendorf tubes and processed using standard in-gel reduction, alkylation and trypsinolysis steps (Shevchenko *et al*., 1996). Samples were transferred to HPLC sample vials and stored at 4°C until LC-ESI-MS/MS analysis. Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromeleon chromatography software (Dionex). A micro-pump flow rate of 246 µl/min^{-1} was used in combination with a cap-flow splitter cartridge, affording a 1/82 flow split and a final flow rate of 3 µl/min^{-1} through a 5 cm x 200 µm ID monolithic reversed phase column (Dionex-LC Packings) maintained at 50°C. Samples of 4 µl were applied to the column by direct injection. Peptides were eluted by the application of a 15 min linear gradient from 8-45% of solvent (80% acetonitrile, 0.1% (v/v) formic acid) and directed through a 3 nl UV detector flow cell. LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (Esquire HCTplus^{TM}, Bruker Daltonics) via a low-volume (50µl/min^{-1} maximum) stainless steel nebuliser (Agilent) and ESI. Raw chromatography data were processed and Mascot compatible files created using DataAnalysis^{TM} 3.2 software (Bruker Daltonics) with the following parameters: compounds (autoMS) threshold 1000, number of compounds 500, retention time windows were 2.0 min for C18 (30 min gradient) and 0.8 min for monolithic and C18 (9 min gradient).

Mascot-compatible files generated were inserted into ProteinScape, version 2.1.0.577 (Bruker Daltonics), and were searched against five databases. First, Nembase Version 4 (http://www.nematodes.org/nematodeESTs/nembase.html), last updated July 2011, which comprises the main depository for information generated from nematode EST databases. The MS data were also searched against an annotated *T. circumcincta* EST database, generated in house at MRI. This EST dataset was derived from two *T. circumcincta* cDNA libraries (one generated from *T. circumcincta* L_3 exsheathed with sodium hypochlorite and one from L_4 obtained at 7 dpi) generated by suppression subtractive hybridisation (SSH) (Nisbet *et al*., 2008). Two of the other databases that were searched were generated by next generation sequencing using Roche-454 titanium technology and these were provided by Dr. Aileen Halliday (MRI) and Professor Steve Paterson (Department of Ecology, Evolution and Genomics of Infectious Diseases, University of Liverpool), respectively. One database was created by 454 sequencing of cDNA derived from RNA obtained from L_3 exposed to either an immune or naive ovine abomasal environment, obtained by the procedures outlined in Halliday *et al*. (2012). More specifically, 100,000 exsheathed *T. circumcincta* L_3 were exposed *in vitro* to fluid collected from the culture of an abomasal fold in saline for 3 h. There were two sources of abomasum; (i) a sheep trickle-infected and bolus-challenged with *T. circumcincta* or (ii) a helminth-naïve sheep (Halliday *et al*., 2012). The other 454 database was created from cDNA generated from RNA from *T. circumcincta* L_4 collected at 7 dpi from a previously uninfected sheep. The final database was created from an EST dataset, this was derived from a *T. circumcincta* cDNA library generated from adult worms which had been harvested from an infected donor sheep (Menon *et al*., 2012).

Database	Reference	Parasite stage	Code
EST data generated from SSH analysis of L_3/L_4	Nisbet *et al*., 2008	L_3/L_4	L_3/L_4 SSH
454 database from L_3 exposed to immune or naïve abomasal environment	Halliday *et al*., 2012	L_3	L_3 Immune - Naive
454 database from L_4 collected at 7 dpi	(Unpublished)	L_4	L_4 454
EST data generated from transcriptomic analysis of adults	Menon *et al*., 2012	Adult	Ad

Table 3.2 Summary of databases for searching against mass spectrometry data.

Interpretation and presentation of MS data were performed in accordance with published guidelines (Taylor and Goodlett, 2005). Fixed and variable modifications selected were carbamidomethyl and oxidation, respectively, and mass tolerance values were set at 1.5 Da and 0.5 Da for MS and MS/MS, respectively. MOWSE scores obtained for individual protein identifications were inspected manually and considered significant only if a) two unique peptides were matched for each protein, and b) each peptide contained an unbroken "*b*" or "*y*" ion series of a minimum of four amino acid residues. Protein identifications were confirmed further with a MOWSE score of 42 or higher indicating that the match was statistically significant at the 95 % confidence level when searching against the four datasets. Proteins identified in the proteomic analysis were analysed further by assessing if the protein was unique to a particular database for example, L_3/L_4 SSH, L_3 Immune/Naive and L_4 454 databases (Table 3.2). All MS analysis was performed at the MRI Proteomics Facility.

3.2.7 Confirmation of the ability of affinity-purified antigens to bind to antibodies in abomasal mucus

To assess if antigens in the affinity-purified L_3 extract bound antibodies from mucus and gastric lymph of previously infected/challenged sheep, 10µl of the affinity-purified L_3 extract were resolved by NuPAGE SDS-gel electrophoresis on a 4-12% gradient gel. The proteins were then transferred to nitrocellulose and the strips blocked as described in section 2.2.5. Blot strips were probed with primary antibody (see Table 2.2 for

dilutions) for 2 h at room temperature. Strips were washed in TNTT for 10 min, three times, and incubated in secondary antibody (Table 2.3) diluted at 1:250 in TNTT for 1 h at room temperature. After re-washing in TNTT, strips were incubated in tertiary antibody (Table 2.3), diluted at 1:1000 in TNTT for 1 h at room temperature. After a final wash in TNTT, strips were incubated in DAB for 6 min in the dark and reactions stopped with several washes in distilled water. At the point between blocking and primary antibody incubation, one strip was treated with sodium periodate (detailed in Section 2.2.7) to disrupt carbohydrate moieties in an attempt to allow comparison between reactivity to protein and carbohydrate in the affinity-purified L_3 extracts.

3.2.8 Measurement of antigen-specific IgA levels in ovine efferent gastric lymph

A direct ELISA was developed to analyse the relationship between binding of IgA present in ovine efferent gastric lymph to IgA-affinity purified L_3 extracts and parameters known to be associated with protective immunity. Affinity-purified L_3 extracts were diluted in ELISA coating buffer (50mM sodium biocarbonate, pH 9.6) to 5 µg/ml and 50 µl/well used to coat 96-well microtitre ELISA plates (Greiner Bio-one, flat bottomed, high-binding plates) overnight at 4°C. Plates were washed six times with PBST (0.05% (v/v) Tween-20) with an automated plate washer (BioTek ELX405). Non-specific binding sites were blocked by incubation with 10% soya milk powder (Infasoy, Cow and Gate) in PBST at 200 µl/well for 2 h at room temperature. After re-washing, plates were incubated with 50 µl of primary antibody. Here, individual efferent gastric lymph samples obtained from (i) trickle-infected/challenged sheep at 7 dpc, (ii) single challenge infected sheep at 7 dpc or (iii) helminth-naïve sheep were used at the primary antibody step. For details of these sample sources see section 2.2.1.2. All gastric lymph samples diluted 1:20 in PBST were incubated for 2 h at 37°C. After washing, 50 µl of secondary antibody (mouse monoclonal anti-bovine/ovine IgA), diluted at 1:250 in PBST, were added for 1 h at 37°C. Plates were washed prior to the application of tertiary antibody (polyclonal anti-mouse immunoglobulins-HRP conjugated) at 50 µl per well, diluted 1:1000 in PBST, for 1 h at 37°C. Plates were

washed, then 50 µl of OPD substrate added per well, and allowed to develop for 20 min at room temperature in the dark. To stop reactions, 25 µl of 2.5M H_2SO_4 were added per well and absorbance read at 492nm. Samples were conducted in triplicate on each plate, and all plates were run in duplicate.

3.2.9 Statistical analysis

Individual ELISA OD values from each infection group were plotted to assess the distribution of the data. As ELISA OD values were not found to be normally distributed, differences between groups were then investigated statistically by a non-parametric Kruskal Wallis test with the post hoc analysis Wilcoxon-Mann-Whitney test (SPSS version 19). Relationships between three immunological parameters, (i) total IgA concentration in efferent gastric lymph at 7 dpc, (ii) total nematode burden and (iii) percentage of inhibited L_4, and the level of IgA binding to the IgA-affinity purified antigens, were investigated by non-parametric correlation analysis using Spearman rank correlation coefficient (SPSS version 19). For all statistical analysis, the level of significance was set at $p<0.05$.

3.3 Results

3.3.1 Purification of IgG from abomasal mucus

Abomasal mucus derived from sheep previously infected with *T. circumcincta* on multiple occasions was used to supply IgA and IgG. Affinity chromatography using Protein G HP agarose columns was used to separate ovine IgG from the samples. A Coomassie stained gel of the unbound and eluted fractions from the chromatography process is shown in Figure 3.2.

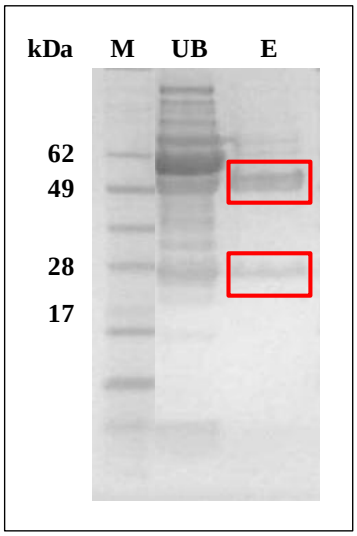

Figure 3.2 SDS-PAGE gel showing purification of IgG from abomasal mucus from previously infected/challenged sheep.

Sheep experimentally previously infected with *T. circumcincta* L_3 were then subjected to a single bolus challenge infection of 50,000 *T. circumcincta* L_3 and necropsied at 2 dpc to collect abomasal mucus. Protein G affinity chromatrography was used to purify ovine IgG. Lane 'UB' contains a sub-sample of the unbound fraction from the Protein G column. Lane 'E' is a sub-sample from the fraction eluted from the Protein G column using 0.1M glycine-HCl, pH 2.5. The eluted fraction contains the heavy and light chains of ovine IgG (highlighted in red boxes). Lane M depicts standard molecular weight markers given in kDa.

Large numbers of proteins were present in the unbound fraction (Figure 3.2, Lane UB). The band displaying the highest intensity of staining is likely to be serum albumin, as the estimated size of the band at approximately 60-62 kDa matches that known for albumin. In the eluted fraction (Figure 3.2, Lane E), there were two separate protein

bands at 28 kDa and 50 kDa, which correspond with the known molecular weights of heavy and light chains of ovine IgG, respectively.

An immunoblot (Figure 3.3) of the unbound eluate from the Protein G agarose column probed for IgA (Lane 1) and IgG (Lane 2) was conducted. Gel electrophoresis was performed under non-reducing conditions to distinguish between IgA and IgG multimer units.

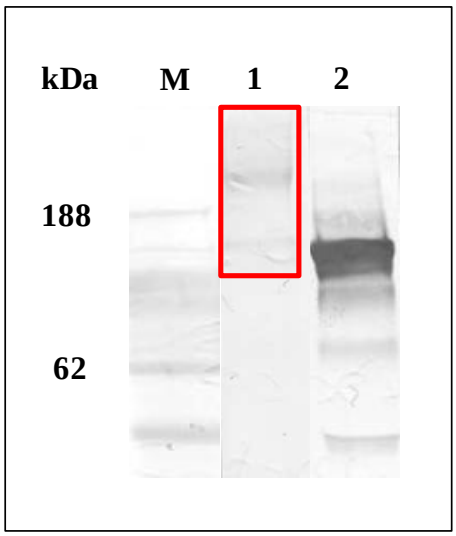

Figure 3.3 Immunoblot of samples obtained by Protein G purification with abomasal mucus from previously infected/challenged sheep.

Lane 1 is the unbound fraction probed for IgA reactivity. An area of IgA binding in the unbound fraction was evident at 120 kDa and above 180 kDa (highlighted by red box). Lane 2 is the unbound fraction probed for IgG reactivity. All strips were incubated with SigmaFast DAB to detect binding of antibodies. Lane M is standard molecular weight markers given in kDa.

The unbound fractions appeared to contain ovine IgA as indicated by binding to the anti-IgA immunoglobulins in the range 120-200 kDa. These sizes correspond with the known high molecular weight multimers of native ovine secretory IgA (Kerr, 1990). The Protein G column depleted, but did not remove all, IgG from the unbound fraction (Lane 2), as indicated by the detection of intense anti IgG-reactive bands at 150 kDa. The binding capacity of the Protein G matrix was 10 mg and it is feasible that the

concentration of IgG in the pooled sample of abomasal mucus could have exceeded this and, as a result, the capacity of the column was saturated.

3.3.2 Size-exclusion chromatographic purification of IgA

The next step was to fractionate the 'unbound' fractions obtained in Section 3.3.1 further by size exclusion chromatography. The chromatographic trace obtained during the size-exclusion purification of IgA shows the absorbance (measured at 280nm) over the complete fractionation process (Figure 3.4).

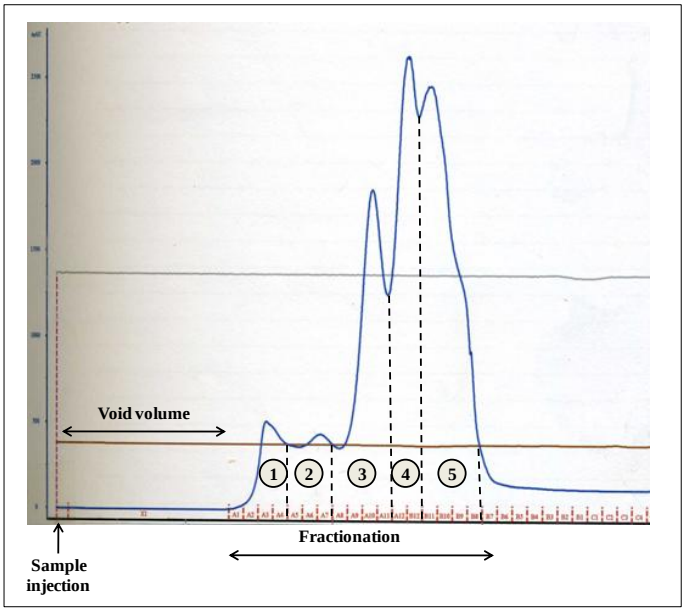

Figure 3.4 Chromatographic trace from the size-exclusion purification of IgA from abomasal mucus obtained from previously infected/challenged sheep.

Samples (200 µl) obtained in Section 3.3.1 were injected at time 0, and the void volume was collected as waste. The blue line is the absorbance at 280nm of the column flow through throughout the process. Fractions 1-5 were collected when the absorbance trace began to increase. Fractions were collected automatically and matched the peaks outlined in the absorbance trace.

The protein profiles of the chromatograph peaks from the fractions collected during size-exclusion purification are shown in Figure 3.5. In the starting material in Figure 3.5 (Lane UB), there are a large number of protein bands present. Secretory IgA is likely to be present in fractions 1 and 2 (Figure 3.5, Lanes 1 and 2), as there is a band at approx

200 kDa (highlighted by the red box). Fractions 3-4 contained high molecular weight host proteins in the range 50-150 kDa. Fraction 5 contained intensely stained protein bands at approximately 30-50 kDa.

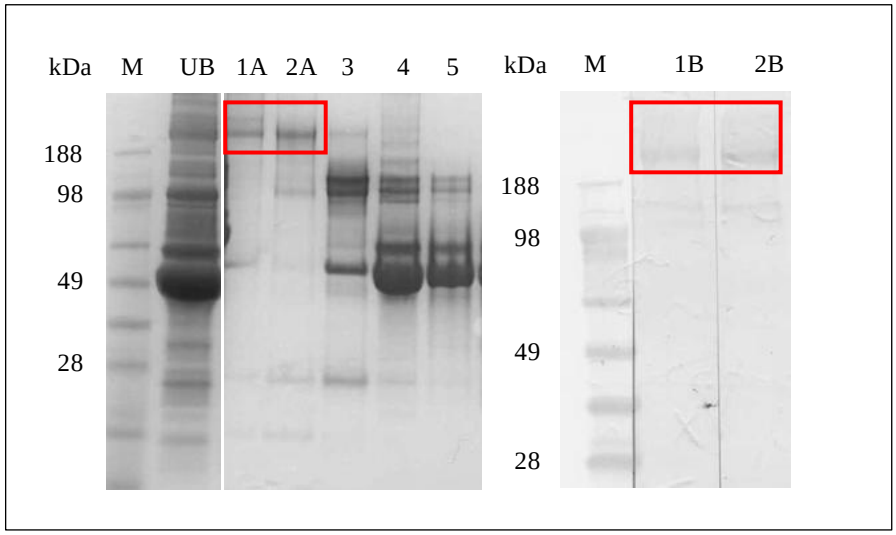

Figure 3.5 Protein profile of ovine IgA from IgG-depleted abomasal mucus by size-exclusion chromatography.

Lane UB is a sub-sample of the starting material of the unbound Protein G fraction. Lanes 1A, 2A and 3–5 are the subsequent fractions following size exclusion separation. Lanes 1B and 2B represent sub-samples from fractions 1 and 2, respectively, probed for IgA reactivity with anti-IgA antibody. The red box surrounds the IgA complex. Proteins are detected by Simply Blue stain. Lane M is standard molecular weight markers given in kDa.

For each of four independent runs, the chromatograph traces exhibited similarities in the absorbance traces, and the protein profiles of the all of the fractions were similar by gel electrophoresis (data not shown). Fraction 1 across all four independent purification experiments contained purified IgA and no other major contaminant bands were detected (Figure 3.5, Lanes 1A and 1B).

Following the two-step purification process, purified IgA was still able to bind to antigens present in *T. circumcincta* L_3 somatic extract: numerous reactive bands were detected in the range of 20–200 kDa (Figure 3.6, Lane 1). Purified IgG bound antigens across the same molecular weight range and showed a region of binding to antigens of high molecular weights (90–200 kDa, Figure 3.6, Lane 3). These immunoblots

confirmed that, following the purification processes for ovine IgA and IgG, the paratopes present on the antibodies that are required for epitope binding appear to be retained.

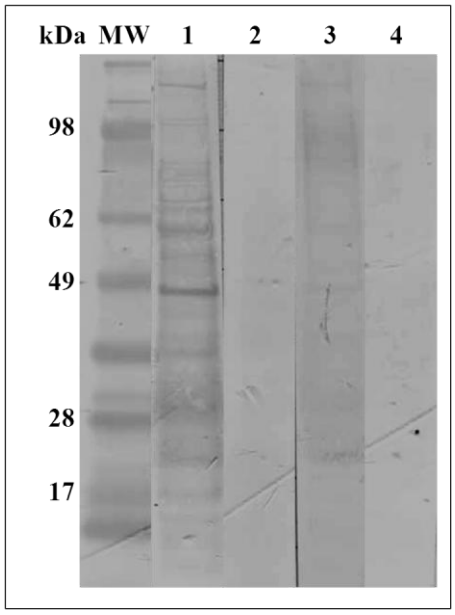

Figure 3.6 Immunoblot of *T. circumcincta* L_3 somatic extract probed with IgA and IgG purified from the abomasal mucus obtained from previously infected/challenged sheep.

L_3 somatic extract (5 µg) was resolved by gel electrophoresis and transferred to nitrocellulose membrane. Lane 1: incubated with purified IgA (see Figure 3.5, lane 1). Lane 2: no primary IgA control. Lane 3: incubated with Protein G purified IgG (see Figure 3.2, lane E). Lane 4: no primary antibody IgG. Lane MW are standard molecular weight markers given in kDa.

3.3.3 Immunoaffinity purification of *T. circumcincta* L_3 somatic antigens

Abomasal mucus IgA from sheep subjected to a trickle infection/bolus challenge protocol purified as described above was used to create an immunoaffinity column for selecting L_3 antigens. Purified IgA was irreversibly chemically cross-linked to NHS-activated sepharose through the antibody's amine bonds. *T. circumcincta* L_3 somatic extract was passed through the custom IgA immunoaffinity column and bound antigens eluted by a gradient of buffers with reducing pH. The protein profiles of the eluted fractions are shown in Figure 3.7.

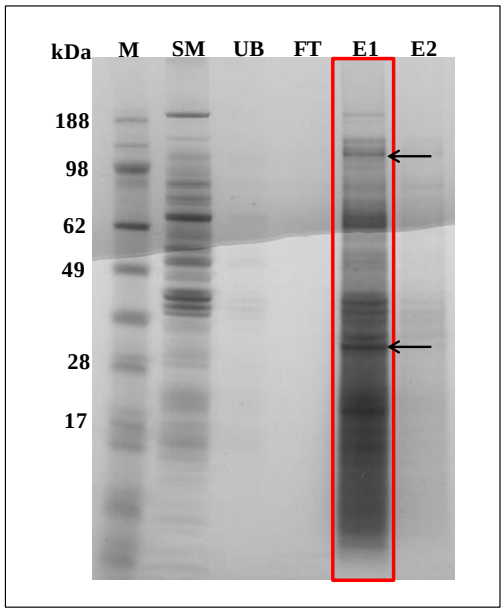

Figure 3.7 SDS-PAGE gel of the fractions collected during immunoaffinity chromatography of *T. circumcincta* L_3 somatic extract using ovine IgA as affinity ligand.

A sub-sample of a L_3 somatic extract (SM) was used for comparison of protein profiles. The unbound fraction (UB) is shown alongside the flow-through (FT) and eluted fractions (E1 and E2). Eluted fractions were collected in 2 column volumes of 0.1M glycine-HCl, 6M urea, pH 2.4. Proteins were stained with Simply Blue stain. Lane M depicts standard molecular weight markers given in kDa. Immunoaffinity chromatography appeared to enrich for proteins at 30 kDa and 100 kDa (highlighted by arrows).

The eluate from the IgA column contained a number of enriched polypeptides over a broad molecular weight range. Immunoaffinity chromatrography with ovine IgA appeared to enrich the *T. circumcincta* L_3 extract for proteins at specific ranges, including 30 kDa and 100 kDa (Figure 3.7, lane E1, highlighted by arrows).

Immunoblotting demonstrated that IgA in gastric lymph from sheep subjected to a trickle infection/bolus challenge protocol bound a range of antigens in the IgA-immunoaffinity purified *T. circumcincta* L_3 extract, with two intensely reactive regions at approximately 20-36 kDa and 60-80 kDa (Figure 3.8, Lane 1). The intensity of IgA reactivity was diminished following periodate treatment of the antigens (Lane 4). Reactivity in the region 60-80 kDa was removed by periodate treatment, while reactivity to the lower molecular weight antigens of 20-40 kDa was retained to a degree (Lane 4). Antigen-specific IgA was not detected in samples obtained from the helminth-free sheep

(Lane 2) and no cross reactivity of the eluted fraction to the developing antibodies was detected (Lane 3).

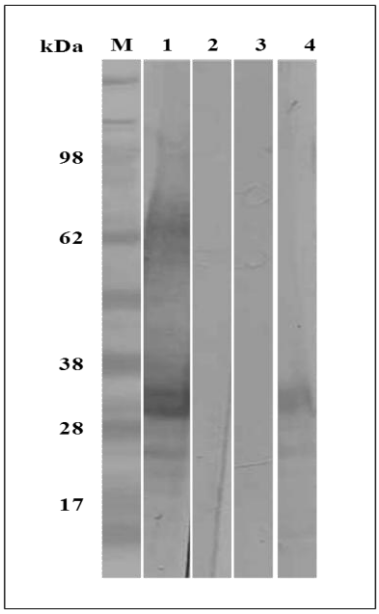

Figure 3.8 Immunoblot of purified IgA-binding antigens from a *T. circumcincta* L_3 somatic extract probed for reactivity to IgA in efferent gastric lymph from previously infected/challenged sheep.

Antigens in the purified IgA-binding fraction from L_3 somatic extract were resolved by gel electrophoresis and transferred to a nitrocellulose membrane for antibody probing. Lane 1, probed with efferent gastric lymph from previously infected/challenged sheep, pooled from samples over 6-10 dpc (*n*=4). Lane 2, probed with efferent gastric lymph pooled from helminth naive sheep (*n*=4). Lane 3, no primary IgA control. Lanes 1–3 were not treated with sodium periodate prior to antibody probing. Lane 4 was treated with 50mM sodium periodate prior to probing with efferent gastric lymph pooled from previously infected/challenged sheep (*n*=4). All lanes were probed with appropriate secondary and tertiary antibodies for IgA detection. Lane M depicts standard molecular weights markers given in kDa.

3.3.4 Protein identification of IgA-immunoaffinity purified L_3 somatic extract

To identify which L_3 somatic extract proteins bound IgA from infected sheep, the eluted fraction from the purification steps was analysed by LC-ESI-MS/MS. The resultant MS data were searched against five databases as described in Section 3.2.6 and results are shown in Table 3.3.

Database	Number of individual peptides identified by LC-MS/M S	Number of proteins with significant matches in each dataset
Nembase	4029	927
L_3/L_4	98	69
L_4 454	2259	2123
L_3 Immune/Naïve	2100	1144
Adult	2952	2570

Table 3.3 Summary of number of peptides and proteins identified through Mascot searches of peptide data.

A total of 374 (containing 155 unique proteins) proteins identified through the proteomic analysis were confirmed after further analysis to ensure that each protein contained two unique peptides with a consecutive sequence of 4 'b' or 'y' ions and had a MOWSE score of >100. Of the 374 proteins, 105 (28.1%) were either hypothetical proteins or their function has not yet been elucidated. The remaining proteins were further classified according to their function. The results are detailed in Appendix 2 and a subset of those proteins of particular interest, on the basis of homology to other parasite vaccine candidates or their putative function at the host/parasite interface, are summarised in Table 3.4. The proteins identified have been divided into a number of functional classes which include: developmental, metabolic, transport, carbohydrate binding, cuticle synthesis, detoxification, lipid binding, heat shock, protein folding, cytoskeletal, actin binding, proteolytic enzymes, signalling and gut-associated (Appendix 2). The number of unique peptides for each protein identified is shown, confirming that each met the minimum criterion of 2 unique peptides.

Protein description	**Organism**	**Accession number**	**MOWSE**	**No. of peptides**	**Estimated mol. weight (kDa)**
14-3-3 like	*C. elegans*	Q20655	372.9	7	56.9
ASP-2	*N. americanus*	AAP41952	166.1	13	26.8
ASP-3 C-type single domain	*O. ostertagi*	CAO00416	100.0	2	79.1
Beta-D-galactosidase	*B. malayi*	AAA27859	367.2	8	66.3
Calponin	*C. elegans*	O01542	201.5	5	43.2
CD109	*A. suum*	ADY40184	162.6	3	74.6
Cytochrome C	*H. contortus*	ACG69807	229.4	5	39.3
DVA-1 polyprotein allergen	*D. viviparus*	Q24702	122.2	2	78.1
Fatty acid/retinol binding protein	*H. contortus*	CDJ96356	272.8	4	49.8
Ferritin	*C. elegans*	CE20622	285.4	5	76.5
Fructose bisphosphate aldolase	*T. circumcincta*	CBO37380	190.5	3	57.1
Galectin 5	*A. cantonensis*	AEK98127	296.4	7	56.2
Galectin 1	*T. circumcincta*	AAD39095	533.1	9	91.4
Glutathione-S-transferase	*H. contortus*	AAF81283	614.7	9	64.1
Intermediate filament protein	*A. suum*	ADY43340	52.6	2	42.5
L3B25	*T. circumcincta*	AAM45145	371.7	7	68.6
Myosin	*C. briggsae*	CAP36983	382.2	5	159.6
Onchocerca related antigen	*C. elegans*	CBO38581	497.8	8	50.9

Paramyosin	*H. contortus*	CBO16022	365.4	6	113.1
Peptidyl prolyl cis-trans isomerase	*O. ostertagi*	P52013	219.1	3	55
Phosphatodylethanolamine binding	*H. contortus*	O16264	247.2	3	19.2
Pterin-4-alpha carbinolamine	*C. elegans*	Q9TZH6	224.7	4	35.7
Putative ES protein F7	*O. ostertagi*	CAD20464	196.0	4	15.3
Putative HEH-1	*C. elegans*	O17271	238.7	4	61.4
Saponin like	*C. elegans*	NP741465	71.3	2	37.3
Superoxide dismutase extracellular	*H. contortus*	P51547	401.1	6	37.5
Thrombospondin	*H. contortus*	AFO43121	468.2	9	171.8
Transthyretin like	*C. brenneri*	EGT36246	209.5	4	56.4
Venom-allergen like (VAL) protein	*C. brenneri*	EGT59294	441.1	9	66.6

Table 3.4 A selection of the proteins identified from proteomic analysis of the IgA-immunoaffinity purified L_3 fraction.

All proteins have been identified by searching MS data against EST databases created from parasite material collected post-infection. Coverage of the full-length protein sequence by the assigned peptides is shown under the percentage of sequence coverage. The protein MOWSE score is shown and all scores are above the threshold and are significant at the 95% confidence threshold. Hits with trypsin and keratin were omitted.

3.3.5 Measurement of IgA levels in ovine efferent gastric lymph to the affinity-purified L_3 extract

To allow investigation of the immunoreactivity of the L_3 antigens eluted from the IgA-affinity column, antigen-specific IgA levels were assessed, in efferent gastric lymph obtained from sheep with varying levels of immunity, by antigen-specific ELISA (Figure 3.9). Mean antigen-specific IgA levels in the gastric lymph of previously infected/challenged sheep, 7 dpc, were significantly higher (p=0.021) than those in sheep subjected to a single infection and in sheep that were helminth-free (Figure 3.9).

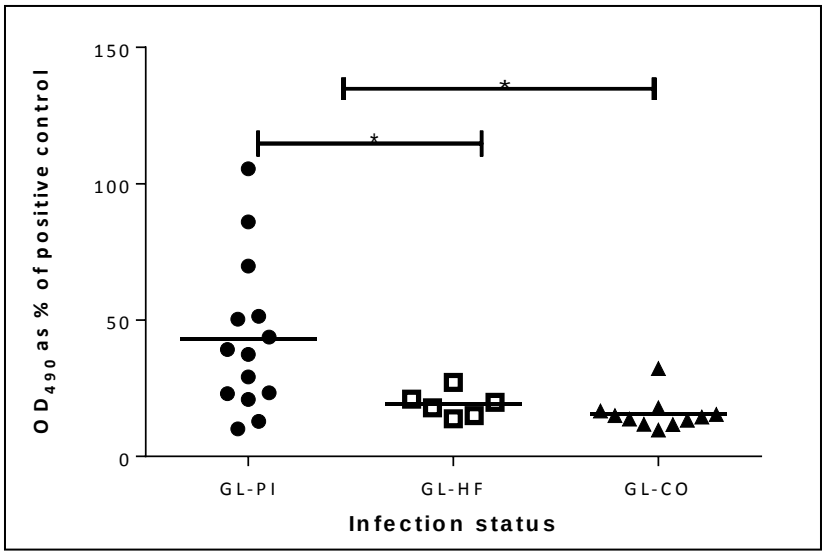

Figure 3.9 Levels of binding of IgA from efferent gastric lymph to antigens present in the IgA-immunoaffinity purified L_3 fraction.

ELISA plates were coated with the IgA-immunoaffinity purified L_3 somatic extract (5µg/ml) and incubated with efferent gastric lymph from individual sheep of different infection status. Group GL-PI: previously infected/bolus challenge (*n*=14). Group GL-CO: single infection *(n*=11). Group GL-HF: helminth-free (*n*=6). Samples from Groups GL-PI and GL-CO were from a single time-point 7 dpc with 50,000 *T. circumcincta* L_3. The level of IgA binding was detected by HRP-labelled developing antibodies. Results are expressed as percentage of the positive control. The positive control was efferent gastric lymph pooled from previously infected/challenged sheep over 6-10 dpc. Samples were conducted in triplicate with two independent repetitions. '*' grouped median IgA concentrations of GL-PI sheep (Group 1) were significantly higher ($p<0.05$) than GL-CO or GL-HF median values.

Relationships between the level of IgA binding and parasitological and immunological parameters were investigated and are summarised in Table 3.5.

Parameter	R_s value	P-value	Significance level
Nematode burden	-0.565	0.004	**
% inhibited larvae	0.534	0.007	**
Total IgA in lymph	0.853	<0.001	***
Blast cell response	-0.058	0.979	NS

Table 3.5 Relationships between antigen-specific IgA levels and parasitological and immune parameters.

A significant negative correlation (p=0.004, r_s = -0.565) was observed between antigen-specific IgA levels in efferent gastric lymph and the total *T. circumcincta* burdens at necropsy (Figure 3.10; Table 3.5). The total burdens were assessed by counting the number of adult males, females and inhibited L_4 in a sub-sample of the digests of abomasal tissue and contents (Halliday *et al*., 2007).

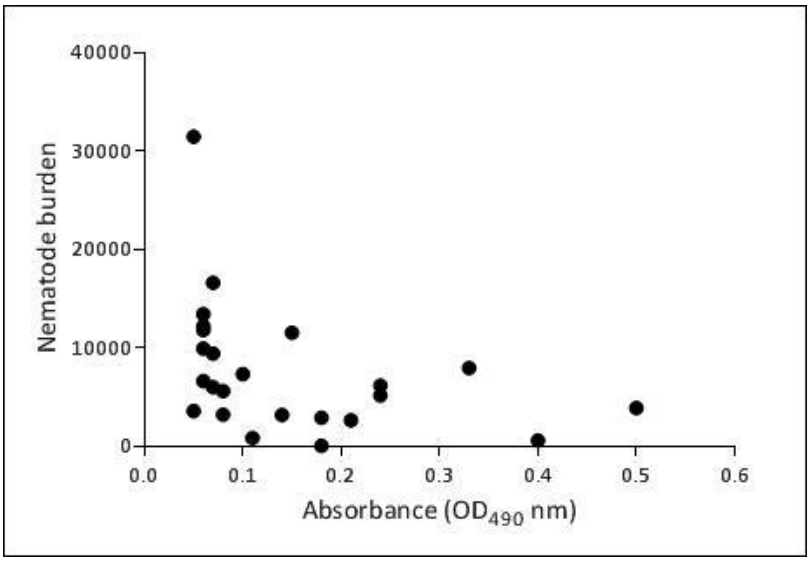

Figure 3.10 Relationship between the level of gastric lymph IgA binding to antigens in an IgA-immunoaffinity purified *T. circumcincta* L_3 somatic extract and the total nematode burden of sheep previously infected/challenged with *T. circumcincta* L_3.

Analysis of the association was conducted using Spearman correlation coefficients. Data points represent absorbance (OD) values from individual gastric lymph samples (*n*=23). Absorbance values were plotted against the total nematode burden for each individual. The efferent gastric lymph was collected from sheep which had been previously infected with *T. circumcincta* L_3 and subjected to a bolus challenge of 50,000 *T. circumcincta* L_3. The gastric lymph samples used were all from a single time-point (7 dpc). The worm burden of the "GL-PI" sheep was assessed by counting the number of males, females and inhibited L_4 in a sub-sample of the digests of the abomasal tissue and contents (Halliday *et al*., 2007).

In contrast, a significant positive correlation ($p=0.007$, $r_s = 0.534$) was observed between the antigen-specific IgA levels in efferent gastric lymph and the percentage of inhibited L_4 enumerated at necropsy (Figure 3.11; Table 3.5). Some of the data points are stated as having 0% of inhibited L_4; these are the samples from helminth-naïve and/or the sheep administered a bolus challenge of 50,000 *T. circumcincta* L_3 only (Figure 3.11).

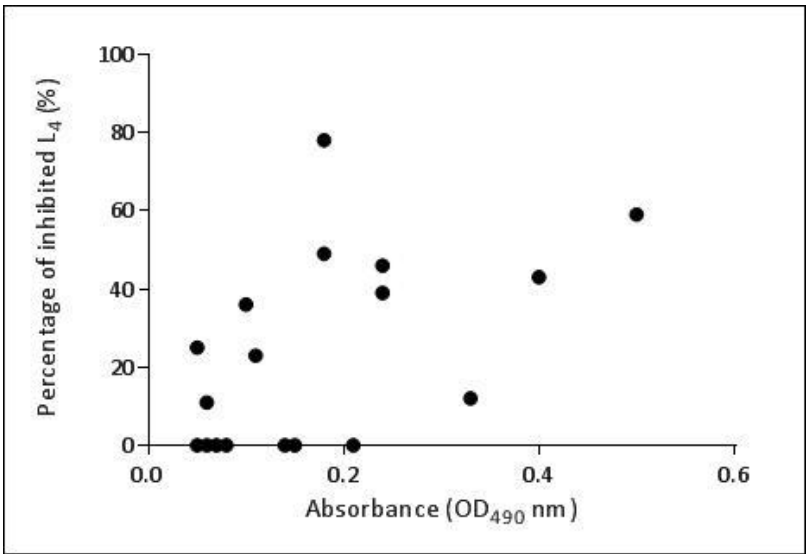

Figure 3.11 Relationship between the level of gastric lymph IgA binding to antigens in an IgA-immunoaffinity purified *T. circumcincta* L_3 somatic extract and the percentage of inhibited *T. circumcincta* L_4 in the abomasa of sheep previously infected/challenged with *T. circumcincta* L_3.

Analysis of the association was as in Fig 3.10. Data points represent the absorbance values (OD) in individual gastric lymph samples (n=19). The absorbance values were plotted against the percentage of inhibited *T. circumcincta* L_4 in the abomasum at post mortem. Efferent gastric lymph was collected from individual sheep as detailed in Fig. 3.10. The percentages of inhibited *T. circumcincta* L_4 present in the abomasum were estimated as detailed in Halliday *et al*. (2007).

A strong positive correlation ($p<0.001$, $r_s = 0.853$) was found between the level of L_3-antigen-specific IgA levels in efferent gastric lymph and the total IgA concentration in the efferent gastric lymph (Figure 3.12; Table 3.5). The correlation analysis of this relationship found it to be tighter than the relationship between the level of L_3 antigen-specific IgA levels and the total nematode burden at necropsy (Figure 3.10).

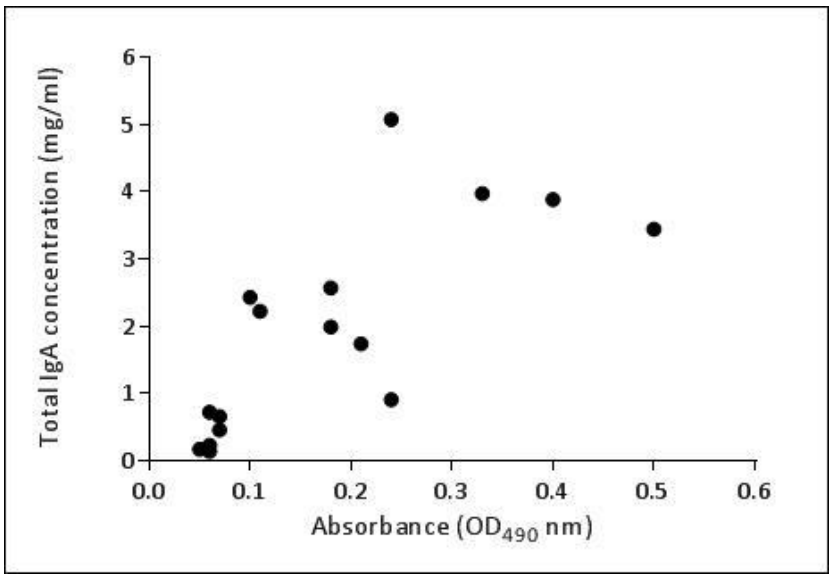

Figure 3.12 Relationship between the level of gastric lymph IgA binding to antigens in an IgA-immunoaffinity purified *T. circumcincta* L_3 somatic extract and the total gastric lymph IgA concentration at 7 dpc of sheep previously infected/challenged with *T. circumcincta* L_3**.**

Analysis of the association was conducted as detailed in Figure 3.10. Data points represent the absorbance values (OD) from individual gastric lymph samples (*n*=16). The absorbance values were plotted against the total IgA concentrations for the individual samples. The efferent gastric lymph was collected from sheep which had been previously infected with *T. circumcincta* L_3 and were subjected to a bolus challenge of 50,000 *T. circumcincta* L_3 ("GL-PI"), as in Figure 3.10. Total IgA concentrations were published previously in Halliday *et al.* (2007).

No significant correlations (P=0.979, r_s = 0.979) were observed when the antigen-specific IgA levels were compared with the number of blast cells present in the lymph at 7 dpc (Table 3.5).

3.4 Discussion

Here, for the first time with *T. circumcincta*, immunoaffinity chromatography was used to identify potentially protective antigens present in *T. circumcincta* L_3 somatic extracts using abomasal mucus IgA. Chromatography methods were used to purify IgA from abomasal mucus from previously infected sheep and the purified IgA was used, via immunoaffinity purification, to enrich a somatic extract of *T. circumcincta* L_3 for a subset of IgA-reactive antigens. The proteins in the subset of IgA-reactive antigens purified from *T. circumcincta* L_3 somatic extract were then identified through proteomic analysis. Proteomic analysis identified a range of possible vaccine candidates which had not previously been identified in L_4 somatic extract and/or L_4 ES products, including: fatty-acid/retinol binding protein (FAR), galectin, paramyosin and activation-associated protein (ASP-1). Antigenic proteins identified from extracts of nematode parasites will not necessarily offer protection if they are used to immunise the host against challenge (Kiel *et al*., 2007). The relative immunoreactivity of antigens in the purified L_3 fraction was investigated by comparing antigen-specific IgA responses in sheep with varying levels of immunity to known parameters of the immune response. Mean antigen-specific IgA levels were significantly ($p<0.05$) higher in the trickle-infected/bolus-challenged sheep compared to sheep subjected to a single bolus infection or sheep that had been reared in a helminth-free environment. Positive associations existed between levels of affinity-purified L_3 antigen-specific IgA and the total IgA concentration in efferent gastric lymph at 7 dpc ($p<0.001$) and the percentage of inhibited L_4 calculated at necropsy 3 days after the lymph samples were collected ($p<0.01$). In contrast, a negative correlation between levels of affinity-purified L_3 antigen-specific IgA and nematode burden was demonstrated ($p<0.01$). Proteomic analysis of the IgA-affinity-purified *T. circumcincta* L_3 antigens was used to identify the proteins present and this yielded candidates of particular interest due to their selection as vaccines for other helminth species (for example paramyosin, ASPs, FARs, and galectin) and/or their putative role at the host/parasite interface (for example superoxide dismutase and glutathione-*S*-transferase). Immunoaffinity chromatography using abomasal mucus IgA to purify

antigens from *T. circumcincta* L_3 somatic extract revealed a number of promising vaccine candidates which are potentially involved in the generation of a protective immune response.

ASP-like and venom allergen-like (VAL) proteins were detected in the IgA-affinity-purified *T. circumcincta* L_3 extract and these may represent key vaccine candidates against parasitic nematodes (Hawdon *et al*., 1996; Nisbet *et al*., 2010a). ASPs are nematode-specific members of a diverse protein family, called SCP/Tpx-1/Ag5/PR-1/Sct (SCP/TAPS), present in eukaryotes and a range of parasites (Cantacessi *et al*., 2009; 2012). The first report of their existence in parasites was in the ES products of serum-activated L_3 of the canine hookworm, *Ancylostoma caninum* (Hawdon *et al*., 1996; 1999). They are key vaccine candidates against hookworm species and are thought to be important in the establishment of parasites in the host (Hawdon *et al*., 1999; Tawe *et al*., 2000).

Here we found for the first time that the affinity-purified *T. circumcincta* L_3 extract contained proteins that showed homology to two ASPs: an ASP-2 from *A. caninum* (Ac-ASP-2) and a C-type single domain ASP-3 from *O. ostertagi* (Oo-ASP-3). Phylogenetic analysis of Ac-ASP-2 and Oo-ASP-3 has shown that they are closely related homologues (Visser *et al*., 2008). A randomised, placebo-controlled, double-blind vaccination trial with an ASP-2 homologue from the human hookworm *Necator americanus* (Na-ASP-2) indicated that immunisation of humans with recombinant Na-ASP-2, injected intramuscularly with an alhydrogel adjuvant, induced antigen-specific serum IgG titres significantly higher than controls up to 8 months after the final vaccination (Bethony *et al*., 2008). Serum antibodies from vaccinated humans were cross-reactive with native Na-ASP-2, prepared from an extract of *N. americanus* L_3 (Bethony *et al*., 2008), indicating that there were structural similarities between the recombinant and native proteins. In a vaccine trial of *A. caninum* in dogs, vaccination with a recombinant version of Ac-ASP-2 was protective against a challenge infection, with 69% and 26% reductions in faecal egg counts and adult worm burdens, respectively, in vaccinated dogs compared to the control group (Bethony *et al*., 2005).

An investigation into the transcription of ASPs in *O. ostertagi* revealed that the transcript of Oo-ASP-3 is expressed in all larval and adult stages, and the protein was detected in the oesophagus and intestines of male and female worms (Visser *et al*., 2008). Oo-ASP was first identified in *O. ostertagi* adult ES products through affinity chromatography to enrich for proteins with affinity to thiol groups (Geldhof *et al*., 2003). It was later revealed to be involved in the protective immune response against infection of cattle with *O. ostertagi*, as vaccination of cattle with the native ASP antigen (purified through affinity for thiol) saw a significant reduction of 74% in cumulative egg output compared to the unvaccinated controls (Meyvis *et al*., 2007). As *O. ostertagi* is a closely related parasite to *T. circumcincta*, this strongly suggested that ASP may also be involved in the protective immune response in *T. circumcincta*-infected sheep. Abomasal mucosal antibodies in *T. circumcincta*-infected sheep are specific to a number of ASPs in L_4 ES products (Nisbet *et al*., 2010a; Smith *et al*., 2009). In the current study we have shown for the first time that the affinity-purified *T. circumcincta* L_3 extract contained a homologue of Oo-ASP-3. This Oo-ASP-3 homologue was different to the homologues previously identified in L_4 ES products (Nisbet *et al*., 2010a; Smith *et al*., 2009). This novel identification of an ASP homologue in a *T. circumcincta* L_3 somatic extract supports the proposed role of ASPs in establishment of infection (Hawdon *et al*., 1996), as the parasite could be producing and sequestering ASP-3 homologues in pre-parasitic larval stages for release upon ingestion to allow establishment and maintenance of the parasite in the host. This is supported by studies with Ac-ASP-2, as transcript analysis revealed that its mRNA is present at all life-cycle stages but protein expression was only detected in L_3 activated with host serum *in vitro* (Hawdon *et al*., 1999).

The ASP-1 homologue found previously in *T. circumcincta*, using the local IgA response (Tci-ASP-1, Nisbet *et al*., 2010a), was not identified in the IgA-reactive L_3 antigens identified in this current study. One of the reasons potentially behind this is that the L_3 somatic extract used this study was prepared from free-living larvae, and the larvae had not been exposed to the host environment. In contrast, in the previous study,

Tci-ASP-1 was identified in larvae which had already established in the host and had been exposed to the host environment, as it was identified in ES products of L_4 collected at 5 dpi. This indicates that there could be a mechanism, following ingestion, which triggers rapid synthesis and release of ASPs following infection, and is supported by studies with Ac-ASP-1 where the majority of the protein is released in the 4 h following stimulation with a low molecular weight ultrafiltrate of host serum and methylated glutathione analogues (Hawdon *et al*., 1996).

The work here identified for the first time other members of the SCP/TAPS protein family, cysteine-rich secretory VAL proteins; these are closely related to ASPs (Mitreva *et al*., 2005). Homologues of VAL proteins have been identified in the ES products of *Heligmosomoides polygyrus* adults (Hewitson *et al*., 2011) and *Brugia malayi* microfilaria and adults (Bennuru *et al*., 2009; Hewitson *et al*., 2008). In *B. malayi*, VAL-1 is a target of the immune response with antigen-specific IgG_3 and IgG_4 antibodies present in 95% of microfilaraemic patients with circulating parasites tested from a panel of human filariasis sera (Murray *et al*., 2001). Vaccination with recombinant VAL-1 in jirds indicated there was a protective response as there was a 64% decrease in the number of parasites recovered post-challenge in immunised jirds compared to the control group (Murray *et al*., 2001).

Immunoreactivity of structural and muscle-derived proteins, including paramyosin, myosin and calponin, during natural exposure to pathogens has been observed previously in other proteomic studies on other helminth species (Bennuru *et al*., 2009; Curwen *et al*., 2004; Kiel *et al*., 2007, Murphy *et al*., 2010). Paramyosin is an α-helical coiled protein and is a component of thick muscular filaments of invertebrate species (Epstein *et al*., 1985). The first report of its immunoreactivity was in mice immunised with *Schistosoma mansoni* adult extracts (Lanar *et al*., 1986) and paramyosin was localised to the tegument muscle layers of adult *S. mansoni*. Paramyosin is now a lead vaccine candidate against a range of parasites including *Brugia malayi* (Li *et al*., 1999; 2004), *Taenia solium* (Vazquez-Talavera *et al*., 2001), *Trichinella spiralis* (Wei *et al*., 2011; Yang *et al*., 2010) and *Schistosoma japonicum* (McManus *et al*., 2001).

Studies in *B. malayi* have used a DNA vaccine encoding paramyosin to assess its potential as a vaccine candidate in mice (Li *et al*., 1999) and jirds (Li *et al*., 2004). Vaccination with plasmid DNA induced strong humoral responses, both IgG_1 and IgG_{2a} responses (Li *et al*., 1999; 2004), but was not protective against challenge infection and induced lower levels of antibody responses compared to vaccination with the native and recombinant versions of the protein (Li *et al*., 1993; 1999). Vaccination against *T. solium* with a recombinant full-length version of paramyosin was protective against challenge infection, with a 52% reduction in the parasite burden of vaccinated mice compared to unvaccinated controls (Vazquez-Talavera *et al*., 2001). This initial study into paramyosin led to further studies into identification of T-cell epitopes with an aim to develop a vaccine (Lopez-Moreno *et al*., 2003). However this research has not to date translated into vaccine development.

In *T. spiralis* vaccine studies, recombinant paramyosin induced significantly higher antigen-specific serum IgG titres in immunised mice compared to unvaccinated control mice and was protective against a challenge infection, with a 37% reduction in worm burdens in vaccinated mice compared to the unvaccinated controls (Yang *et al*., 2010). Recently, attempts were made to identify the epitope on paramyosin which is essential for antibody binding (Wei *et al*., 2011). Also, paramyosin expression in both larval and adult *T. spiralis* was targeted by RNA interference and resulted in a reduction in viability of the parasites (Chen *et al*., 2012). Collectively, the studies into the suitability of paramyosin as a protective antigen against a range of parasites strongly suggest that it is a possible vaccine candidate against *T. circumcincta* L_3.

Recently paramyosin has also been identified in parasitic helminths, including *T. colubriformis* (Kiel *et al*., 2007), *T. circumcincta* (Murphy *et al*., 2010) and *D. viviparus* (Strube *et al*., 2009). Using a 2-dimensional PAGE gel probed with serum from sheep with a high immunological responsiveness to *T. Colubriformis*, it was shown to be highly immunoreactive with serum IgG (Kiel *et al*., 2007). Murphy *et al.* (2010) identified paramyosin in a *T. circumcincta* L_3 somatic extract using a proteomic analysis of a 2-dimensional PAGE gel. Immunoblotting of the 2-dimensional gel with serum

from lambs naturally infected with *T. circumcincta* revealed that serum IgE recognised the antigen (Murphy *et al*., 2010). However, none of the studies detailed above conducted any protection experiments to investigate the potential of paramyosin to protect against parasite infection. In this thesis, paramyosin was detected in the immuno-purified fraction and, as it was purified through affinity for IgA, this further supports its role as a target of the ovine immune system during infection with *T. circumcincta*. Paramyosin from a range of parasitic nematode species is bound by various antibody isotypes: IgG (Loukas *et al*., 2001; Strube *et al.,* 2009), IgE (Murphy *et al*., 2010) and IgA (Hernandez *et al*., 1999; Nara *et al*., 2007). As paramyosin is bound by different antibody isotypes, this could be a result of repeated regions of hydrophobic and charged amino acids conferring strong binding affinity for different isotypes (Gobert and McManus, 2005). Here, we identified paramyosin in a subset of IgA-reactive antigens from *T. circumcincta* L_3.

A homologue of a fatty-acid/retinol binding protein (FAR) was also for the first time found to be present in the IgA-affinity-purified *T. circumcincta* L_3 extract (Table 3.3). FARs are a nematode-specific class of molecules that have selective binding affinity for lipids and have been described from *Onchocerca volvulus* (Kennedy *et al*., 1997), *B. malayi* (Garofalo *et al*., 2002), *H. polygrus* (Hewitson *et al*., 2011), *N. americanus* (Daub *et al*., 2000) and *A. caninum* (Basavaraju *et al*., 2003). Fatty acids and retinol (vitamin A) are essential for parasite development (Kennedy *et al*., 1997; Wolff and Scott, 1995); however, as parasites do not possess pathways for synthesis of these molecules (Behm, 2002), they must be able to acquire them from the host. The function of FARs in nematodes is not yet clear but they have been proposed to play a role in host-parasite interactions. The potential role could involve scavenging retinol produced by the host, which in turn may promote or favour parasite survival in the abomasum as this depletion of retinol can then impair the level of efficacy of the host's Th2 immune response (Basavaraju *et al*., 2003). Retinol is thought to have a key role in the hosts' immune response in the local environment infected by the parasite as it has been shown to be involved in the repair of gut tissue damage (Bulger and Helton, 1998)

and in the prevention of the decline of secretory IgA in the small intestine of mice (Nikawa *et al*., 1999). So, therefore, a mechanism by which the parasite can bind retinol may be an immune evasion strategy as it reduces the level of protective IgA in the mucosa. Two types of FAR were identified in *A. caninum* through immunoscreening of a cDNA library with antibodies against *A. caninum* adult ES products (Basavaraju *et al*., 2003). However, despite mRNA encoding these molecules being present in L_3 *A. caninum*, there were no detectable corresponding proteins expressed in L_3 stages, in contrast to the results from *T. circumcincta* L_3 here. In *O. ostertagi* a FAR homologue was only detected, by immunoscreening, in the ES released from L_4 and adult but not L_3 stages (Vercauteren *et al*., 2003). The immunoaffinity screening approach used in this thesis may have enriched the FAR to such an extent that it was detectable in L_3 stages whereas previous studies may have been insufficiently sensitive to detect the protein.

Two antioxidants were identified in the IgA-affinity-purified *T. circumcincta* L_3 extract; extracellular superoxide dismutase and glutathione-*S*-transferase. Antioxidants scavenge free radicals produced during oxidative stress and, in turn, could have a key role in parasite survival. Proteomic analysis of ES products from adult *H. contortus* identified both extracellular superoxide dismutase and glutathione-*S*-transferase; however, these molecules were not found to be immunoreactive with sera from hyperimmune sheep (Yatsuda *et al*., 2003). The antioxidant activity of superoxide dismutase has been noted as higher in L_3 than in adult stages of a number of gastrointestinal parasitic nematode species, including *T. circumcincta* (Hadas and Stankiewicz, 1998; Knox and Jones, 1992). As these were pre-parasitic stages this suggests that the antioxidant capacity of superoxide dismutase is required during interactions with the host, to counteract the oxidative stress associated with the mucosal inflammatory response during parasite invasion and to allow development through its life cycle stages (Knox and Jones, 1992). High levels of superoxide dismutase activity were seen in soluble antigen extracts of both *D. viviparus* L_3 and adults, as well as adult ES products (Britton, Knox and Kennedy, 1994). Incubation of these extracts with serum IgG (purified from calves either naturally infected with the parasite through a

field challenge or from calves vaccinated with 20,000 irradiated L_3) showed that superoxide dismutase activity was inhibited by the antibodies (Britton, Knox and Kennedy, 1994). The identification of superoxide dismutase in the IgA-immunoaffinity purified *T. circumcincta* L_3 fraction supports its possible roles in establishment in the host. Immunoreactivity of helminth glutathione-*S*-transferases has been shown, using a 2-dimensional gel electrophoresis and western blotting approach, in which extracts of both adult *F. hepatica* and *S. mansoni* were probed for serum IgG reactivity using a pool of serum samples which were seropositive for schistosomiasis and fascioliasis (Boukil *et al*., 2011). Here, the proteomic identification of glutathione-S-transferase and superoxide dismutase in the IgA-reactive *T. circumcincta* L_3 antigens suggests that these antigens are targets of the immune response and could confer protection against infection in a novel vaccine directed towards incoming L_3.

The theory behind the immunoaffinity purification process used in this Chapter was to allow identification of IgA reactive antigens under native conditions. Following periodate treatment of the IgA-immunoaffinity purified *T. circumcincta* L_3 extract (see Figure 3.8) the reactivity of the antigenic bands at approximately 60 kDa was depleted, suggesting that these molecules may contain carbohydrate domains. The protein identified in the IgA-immunoaffinity purified *T. circumcincta* L_3 extract with the highest MOWSE score was the glycoprotein thrombospondin. This is a component of the lead vaccine candidate complex from *H. contortus*, H-gal-GP from adult worms, but its transcript was not detected in sheathed or exsheathed *H. contortus* L_3 stages (Skuce *et al*., 2001). Thrombospondin contains numerous potential glycosylation sites; evidence shows that the backbone of a thrombospondin-like immunodominant antigen from *D. viviparus* is attached to N-linked glycans and is highly immunogenic (Kooyman *et al*., 2009).

Galectins are a lectin family with high affinity for β-galactosides and have been identified in a number of helminth parasites; *H. contortus* (Greenhalgh *et al*., 2000; Newlands *et al*., 1999), *O. volvulus* (Klion *et al*., 1994), *T. circumcincta* (Greenhalgh *et al*., 1999) and *T. colubriformis* (Greenhalgh *et al*., 1999). The identification of a

galectin in extracts of *T. circumcincta* L_3 is consistent with findings reported by Newton *et al*. (1997). In immunoproteomic studies of *T. colubriformis*, two galectin homologues were identified in L_3 extracts and these were reactive against serum IgG from sheep with high immunological responsiveness to *T. colubriformis* (Kiel *et al*., 2007). The developmental regulation of three galectins in the parasitic nematode, *H. contortus*, indicated differences in the levels of expression across all life cycle stages (Greenhalgh *et al*., 2000). Differences in the patterns of galectin expression in *H. contortus* L_3 were evident; galectin-1 and galectin-4 showed increased expression in sheathed L_3 compared to the other stages (Greenhalgh *et al*., 2000). L_3 used to prepare the somatic extract used in the immunoaffinity purification in the current study were also sheathed. The roles of galectins in parasitic nematodes are not fully understood, but there are suggestions that they modulate the host's immune response through mimicking host galectins to allow the parasite to evade the immune response (Vasta, 2009; Young and Meeusen, 2004).

Transthyretin-like proteins (TTLs) were also identified in the IgA-affinity-purified *T. circumcincta* L_3 extract. TTLs have an affinity for small hydrophobic ligands, and have been identified in ES products from adult extracts of *B. malayi* (Hewitson *et al*., 2008), *H. polygyrus* (Hewitson *et al*., 2011), *H. contortus* (Cantacessi *et al*., 2011; Yatsuda *et al*., 2003) and *O. ostertagi* (Saverwyns *et al*., 2008). They were identified as highly immunoreactive against serum IgG obtained from sheep rendered hyperimmune to *H. contortus* infection by an experimental infection model (Yatsuda *et al*., 2003). A recombinant TTL was used in a vaccination trial in dogs against *A. caninum* and significant negative correlations were found between antigen-specific serum IgE levels and worm burdens (Hotez *et al*., 2003). It is suggested that TTLs could bind the hosts' version of retinoic acid (Hewitson *et al*., 2008; 2009). As retinoids (for example vitamin A) have been implicated in the co-induction of regulatory T-cells alongside transforming growth factor (TGF-β) (Mucida *et al*., 2007), the TTLs could enhance the sequestration of vitamin A by the parasite and allow the parasite to evade the host's immune response.

An immunophilin, peptidyl-pro-cis-trans isomerase, was also identified in the *T. circumcincta* L_3 extract. Homologues have been previously identified in *B. malayi* ES products from moulting L_3 (Bennuru *et al*., 2009) and *B. malayi* adult extracts (Hewitson *et al*., 2008). This protein is involved in protein folding and cuticle synthesis (Fischer and Aumuller, 2003). Phosphatidylethanolamine binding protein is potentially involved in the cuticle moulting step, as its expression was upregulated in moulting L_3 of *B. malayi* (Bennuru *et al*., 2009). The glycolytic enzyme identified here in IgA-immunoaffinity purified *T. circumcincta* L_3 extract, fructose bisphosphate-1,6-aldolase, has previously been found in *O. volvulus* L_3 (McCarthy *et al*., 2002) and extracts of adult *S. mansoni* (El-Dabaa *et al*., 1998). Immunolocalisation experiments revealed that antibodies generated against the enzyme bound to the region where the cuticle separates during the moulting process in *O. volvulus* (McCarthy *et al*., 2002), and highlights the potential for this as a novel vaccine candidate directed at incoming *T. circumcincta* L_3.

Although 353 proteins were identified from the proteomic analysis of the IgA-affinity-purified *T. circumcincta* L_3 extract, there were a considerable number of peptides which were not assigned identities or proteins with unknown functions. The genome sequencing project for *T. circumcincta* is not yet complete (as of September 2012;http://www.sanger.ac.uk/resources/downloads/helminths/teladorsagiacircumcincta.html) and it is likely that sequence coverage of the selected proteins will be improved on completion of the genome initiative.

Antibodies generated against the L_3 stage of *T. circumcincta* are thought to be associated with the protective immune response against challenge (Stear *et al*., 1995), with reports of reductions in worm length in the trickle-infected/bolus-challenged group compared to the group infected with a single bolus challenge (Halliday *et al*., 2007; Sutherland *et al*., 1999). The potential role of IgA-affinity-purified *T. circumcincta* L_3 extract in protection against infection with *T. circumcincta* was assessed here by comparing antigen-specific IgA responses present in efferent gastric lymph from individual sheep with the following parameters; total *T. circumcincta* burden, total number of inhibited L_4, total IgA concentration in efferent gastric lymph and the local

specific lymphoblast response (Halliday *et al*., 2007). This investigation revealed that antigen-specific IgA levels were significantly higher in gastric lymph from sheep rendered experimentally immune via a trickle infection/bolus challenge compared to those infected with a single bolus challenge or helminth-naive sheep. This was also reflected in the IgA levels of these samples, directed against L_4 antigens (Halliday *et al*., 2007). The efferent gastric lymph samples used here were from 7 dpc. This time-point corresponded to the peak of total IgA concentration measured in these efferent gastric lymph samples (Halliday *et al*., 2007). A strong positive correlation between the total IgA concentration in gastric lymph at 7 dpc and binding of IgA to the purified L_3 antigens was found here. This strongly suggests that the antigens present in the IgA-reactive L_3 fraction could be ideal candidates for the development of a vaccine targeted towards incoming L_3 into the abomausm. The correlation between the level of IgA binding to the antigens and nematode burden also suggests that they could be protective against infection.

Both L_3 and L_4 somatic antigen-specific IgA levels in abomasal mucosa have been found to have an inverse relationship with the length of *T. circumcincta* L_4 recovered from the abomasum (Stear *et al*., 1995; 1999; 2004; Strain and Stear, 1999). In the study from which the gastric lymph samples used in this Chapter were obtained (Halliday *et al*., 2007), the authors concluded that the peak in IgA concentration in the lymph occurred too late to be associated with the parasite loss and arrested development that was seen by 5 dpc. Here, we report a strong positive association between IgA levels against affinity-purified *T. circumcincta* L_3 extract and the percentage of inhibited L_4 found post mortem in the gastric lymph samples used in that study (Halliday *et al*., 2007). This is consistent with findings from an experimental infection study, where a positive correlation was found between levels of anti-*T. circumcincta* L_3 circulating serum IgA and an increase in the frequency of inhibited L_4 (Beraldi *et al*., 2008). In addition, the levels of circulating serum IgA generated against *T. circumcincta* L_3 whole worm extracts were correlated with a reduction in total adult worms at necropsy (Beraldi *et al*., 2008). Results from Chapter 2 indicated an anamnestic IgA response in efferent

gastric lymph from sheep subjected to a trickle infection/bolus challenge model, which was directed against L_3 somatic antigens, with a peak in antigen-specific IgA levels between 4-7 dpc. Taken together, these findings suggest that local IgA directed against L_3 antigens could be implicated in control of *T. circumcincta* in addition to the immediate hypersensitivity response proposed to mediate exclusion/expulsion of L_3 from the abomasum (Seaton *et al*., 1989; Smith *et al*., 1984).

The large number of proteins identified here highlights the complexity of the B-cell response against complex metazoan parasites and raises the possibility that immune responses directed against multiple antigens may be the most successful approach to vaccination. Further work will be necessary to distil any protective antigens from the suite of L_3 immunogenic antigens identified here. Here, the antigens were identified under native affinity purification conditions to retain post-translational modifications. Previous studies in *O. ostertagi* have reported difficulties in stimulating the same levels of protective immunity with the recombinant version compared to the native version of the protein (Geldhof *et al*., 2008; Meyvis *et al*., 2008), and have linked this failure to the expression systems used to generate the protein. If any of the antigens identified here are to be expressed in recombinant form, the *C. elegans* expression system might be the most appropriate to produce recombinant proteins with appropriate postr-translation modifications; for example, glycosylation (Murray *et al*., 2007). Here, we have identified a number of IgA-reactive L_3 antigens from a *T. circumcincta* L_3 somatic extract. The relative immunoreactivity of antigens in the purified L_3 fraction was investigated by comparing antigen-specific IgA responses in sheep with varying levels of immunity to known parameters of the immune response and revealed that these antigens could be implicated in the generation of a protective immune response mediated by abomasal mucus IgA and directed at regulation of total nematode burden, either through inhibition of development or prevention of establishment.

Chapter 4 : Structural epitopes of *Teladorsagia circumcincta* L_3 somatic antigens

4.1 Introduction

A favoured approach in the development of vaccines against gastrointestinal parasitic nematodes has been the production of recombinant sub-unit protein-based vaccines (Emery *et al*., 1993; Geldhof *et al.*, 2008; Munn, 1997). Recombinant sub-unit vaccine candidate molecules represent native antigens that have been selected on the basis of either presumed functional importance to parasite survival (for example, enzymes for digestion), or on the basis of immunogenic properties, including the induction of host protective immune responses against parasite challenge (Emery *et al*., 1993). However, despite years of research into the development of recombinant subunit vaccines against gastrointestinal parasites, there have been few successes (Geldhof *et al*., 2008; Knox, 2000). Formation of the correct structural epitopes in vaccine candidate molecules has been demonstrated to be crucial (Munn *et al*., 1997; Smith and Smith, 1996; Smith *et al*., 2000). The main hurdle restricting development of recombinant vaccines is thought to be the expression systems used for antigen production (as reviewed in Geldhof *et al*., 2007). These can lead to inadequate or inappropriate post-translational modifications (PTMs) (for example, tertiary protein folding) and consequently can result in the incorrect presentation of structural epitopes required for antibody binding (Geldhof *et al*., 2007). Inappropriate or absent protein glycosylation represents a further important and potentially limiting PTM of recombinant proteins. Novel glycosylation profiles have been identified in one of the lead vaccine candidates against the abomasal parasite, *Haemonchus contortus* (Haslam *et al*., 1996). The glycoprotein vaccine candidate, H11, was isolated from the intestinal brush border of adult parasites and was found to have unusual highly fucosylated N-linked glycans – a profile which has not been described in any other eukaryotic system (Haslam *et al*., 1996). The bacterial expression systems currently used for the production of many recombinant proteins do not facilitate glycosylation of proteins and insect and yeast systems are not able to introduce post-translational modification of the glycans appropriately (Knox and Redmond, 2006;

Nyame *et al*., 2004). Vaccination trials with *H. contortus* compared the protective capacity of both native and bacterial-expressed recombinant forms of cysteine proteases (Redmond and Knox, 2004; 2006), which had been previously targeted by thiol sepharose affinity chromatography of *H. contortus* adult extracts (Knox *et al*., 1995; Knox, Smith and Smith, 1999). Sheep immunized with native cysteine proteases had reductions in faecal egg output and worm burdens of 48% and 46%, respectively, compared to unvaccinated controls (Redmond and Knox, 2004). In contrast, the bacterial-expressed versions gave 38% and 27% reductions in worm burden and faecal egg output, respectively (Redmond and Knox, 2004; 2006). The suggested reason behind the failure of the recombinant to reproduce the same level of protection as the native antigen, was that some of the recombinant proteins expressed by bacterial systems are insoluble and/or lack appropriate glycosylation. Furthermore, re-solubilisation of the antigen can result in problems with incorrect epitope structures. Yeast and eukaryotic expression systems may have the capability to glycosylate the recombinant protein but these can differ from the parasite glycans. A prime example of this is from a vaccination trial against *Fasciola hepatica*. Two different recombinant systems were used to produce versions of a cathepsin L protease, the yeast-expressed version was found to be hyperglycosylated, potentially masking epitopes, whereas the bacuolvirus-produced version was glycosylated and gave a reduction of 52% in fluke burden (Reszka *et al*., 2005).

An alternative, emerging approach is the production of synthetic peptide vaccines based on relevant immunogenic epitopes of protective antigens (Arnon *et al*., 1991; 2000). Synthetic peptide vaccines can be used to prime the host immune response in the appropriate direction, for example, cellular or humoral response, depending on the selection of specific T-cell and B-cell epitopes (Arnon *et al*., 2000). Often, synthetic peptide vaccines are based on epitope mapping of larger immunogenic antigens, to generate small linear amino acid sequences to induce a protective immune response (Arnon *et al*., 1991; 1996). However, as discussed in Chapter 3, discontinuous or structural epitopes have an important role in protective immunity against nematodes.

The identification and synthesis of peptides representing discontinuous epitopes is not straightforward due to the principle that structural epitopes are formed by amino acid residues on separate areas of the antigen which, when brought into close proximity during tertiary protein folding, form a three-dimensional epitope (Goldsby *et al*., 2003). One approach used in an attempt to identify the structure of discontinuous epitopes involves panning of combinatorial random peptide phage display libraries (Coley *et al*., 2001; Fu *et al*., 1997; Gazarian *et al*., 2003; Theisen *et al*., 2000). Peptide sequences identified from such libraries do not necessarily share sequence homology with the primary amino acid sequence of the original antigens; however, they may contain structures that mimic those of structural native epitopes (Arnon, 2000; Smith and Scott, 1993). Phage display could therefore provide a useful tool to identify peptide sequences that mimic the structure of antigenic epitopes, allowing the production of a novel vaccine that can be produced cheaply in sufficient quantities for commercial exploitation (Devlin *et al*., 1990; Sidhu and Koide, 2007).

Phage display libraries contain filamentous bacteriophage, which are viruses that use bacteria as replication vectors; these have been manipulated through cloning of specific DNA sequences into the DNA- or RNA-based genomes (Parmley and Smith, 1988). The inserted DNA sequence is incorporated into a gene encoding one of the bacteriophage minor coat proteins, and the peptide sequence encoding the inserted DNA sequence is therefore displayed on the surface of the bacteriophage (Gao *et al*., 2010). Phage display libraries of random peptides are available commercially to study protein interactions, for example antibody-antigen, and are available with different lengths of displayed peptide sequences, for example 7-mer or 12-mer peptides. The 'biopanning' procedure illustrated in Figure 4.1 involves the co-incubation of an immobilised antibody 'target' with the phage display library. Following several rounds of biopanning, peptides that bind to the antibody with the highest affinity and specificity are identified by sequencing the DNA inserts of the selected clones (Jefferies, 1998). This provides a link between the phenotype of the peptide sequence with the genotype of the inserted DNA sequence.

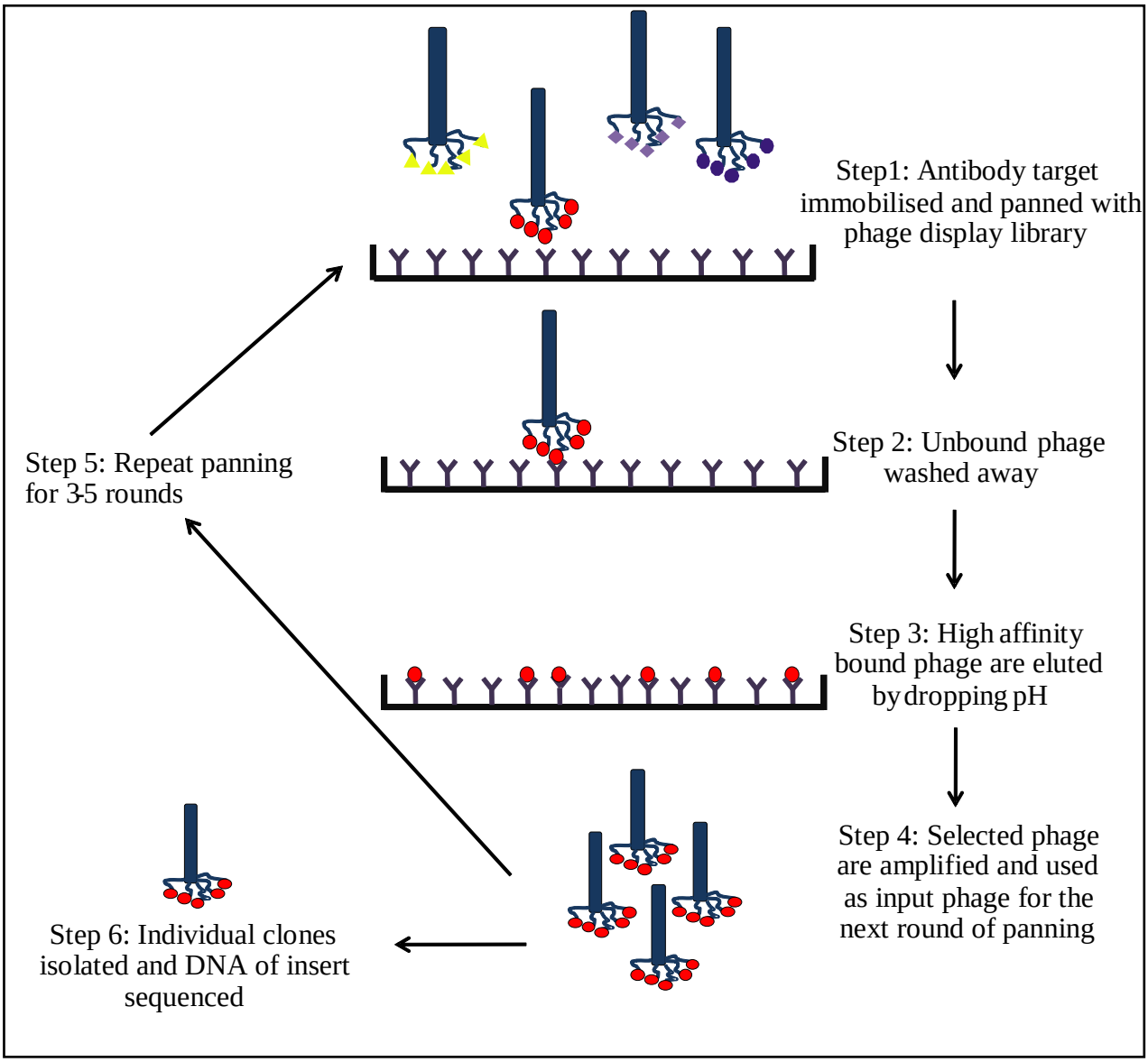

Figure 4.1 Schematic illustration of process of biopanning an antibody target against a random peptide phage display library.

In parasite vaccine development, examples of the use of phage display libraries include schistosomiasis (Adda *et al.*, 1999; Wu *et al.*, 2006), *Trichinella spiralis* (Gu *et al.*, 2008) and *Fasciola hepatica* (Villa-Mancera *et al.*, 2008). One of the studies into vaccine production against *T. spiralis* involved biopanning of a 12-mer phage display library with a monoclonal antibody specifically raised in mice against a 38 kDa antigen found in adult ES products (Gu *et al.*, 2008). Four phage clones selected through

panning were used in immunisation trials in pathogen-free mice (Gu *et al*., 2008). The mice were immunised with individual phage clones or a combination of clones and two of the clones induced antibodies which cross-reacted with a recombinant version of the 38 kDa antigen. Immunisation of mice with these two phage clones, followed by an oral challenge with 400 *T. spiralis* larvae 2 weeks after the final immunisation, induced a reduction of 29% in worm burden compared to unvaccinated animals (Gu *et al*., 2008). Research into the use of phage display libraries in the production of a vaccine against *F. hepatica* used polyclonal antibodies raised in sheep that had previously been immunised with a lead vaccine candidate, cathepsin-L-like cysteine protease (Villa-Mancera *et al*., 2008). Seven 12-mer phage clones derived from panning these libraries with these sera were administered as a multivalent vaccine to yearling sheep, which were then challenged with *F. hepatica* metacercariae. The results showed higher ES-specific serum IgG levels and 34% reduction in fluke burdens in vaccinated animals compared to unvaccinated controls (Villa-Mancera *et al.,* 2008).

In this Chapter, the aim was to use phage display libraries to identify peptide sequences that mimic structural epitopes of *Teladorsagia circumcincta* L_3 antigens. To achieve this, abomasal mucus from sheep, which had previously been exposed to the parasite, was enriched for IgA antibodies that bound *T. circumcincta* L_3 somatic antigens. This was achieved via a two-step immunoaffinity process. The purified antibodies were then incubated with a 7-mer random peptide phage display library to enrich the phage pool for clones with high affinity and specificity for the ovine IgA target. The relative immuno-reactivity of the selected peptide sequences from the phage clones was then assessed by examining antibody reactivity to them using samples from sheep that had acquired immunity to *T. circumcincta* infection through an experimental infection programme.

4.2 Materials and methods

4.2.1 Affinity purification of ovine abomasal antibodies against *T. circumcincta* L_3 somatic antigens

An affinity purification procedure was conducted to purify ovine abomasal mucosal antibodies that bound *T. circumcincta* L_3 somatic antigens. Approximately 450µg of *T. circumcincta* L_3 somatic extract were separated on an SDS-PAGE 4-12% NuPAGE® Bis-Tris Mini gel and transferred to a PVDF membrane (Immobilon, Millipore) as described in section 2.2.7. The membrane was then incubated for 2 h at room temperature in blocking buffer (TNTT , pH 7.4) to block non-specific binding of proteins before washing (x3) in fresh TNTT, 10 min per wash. After washing, the membrane was incubated with a 10 ml pool of abomasal mucus from "Previously infected/challenged" sheep (they had been trickle-infected with 2,000 L_3 three times per week for 8 weeks, then subjected to a bolus challenge of 50,000 L_3; termed 'M-PI", see Table 4.1 for further details) diluted 1:5 in TNTT (Appendix 1) and incubated overnight at 4°C. The membrane was re-washed (x3) in TNTT. To elute bound antibodies, the membrane was incubated with 10 ml of elution buffer (5 mM glycine, 0.5M NaCl, pH 2.5) for 5 min. The eluate was removed from the membrane, neutralised immediately with 500 µl of 1M Tris (pH 8.5) and stored at -20°C. Ovine antibody probes used in this Chapter were the same as described in sections 2.2.1.1 and 2.2.1.2. The abomasal mucus and efferent gastric lymph probes and the associated infection status of the sheep used to generate the antibody probes are detailed in Table 4.1.

Sample	**Infection status**	**Abbreviation**
Abomasal mucus	Trickle infection/challenge Primary infection (challenge only)	M-PI M-CO
Efferent gastric lymph	Trickle infection/challenge Primary infection (challenge only) Helminth-naive	GL-PI GL-CO GL-N

Table 4.1 Summary and abbreviations for samples used in immunoreactivity investigations

Key: M = Abomasal mucus; GL = Efferent gastric lymph; PI = Previously infected by a trickle infection/challenge; CO = Primary infection with a single bolus challenge; N = Helminth-free naive sheep

The antibody-containing fraction eluted from the L_3 immunoblot was concentrated to 1 ml at 4°C using an Amicon centrifugal device Ultra-15 with a 10 kDa molecular weight cut-off (MWCO) membrane. The protein concentration of the eluate was determined using a BCA protein assay kit (Pierce, Thermo Scientific Ltd; section 2.2.4). To purify antibodies which bound glycans on *T. circumcincta* L_3 antigens, this eluted fraction was then incubated with a periodate-treated immunoblot of *T. circumcincta* L_3 somatic extract (sections 2.2.7 and 2.2.7.1). The unbound fractions in this step are likely to contain antibodies which would normally bind glycans, as the structures of their corresponding antigenic epitopes will have been distorted by sodium periodate treatment, preventing binding (Figure 4.2).

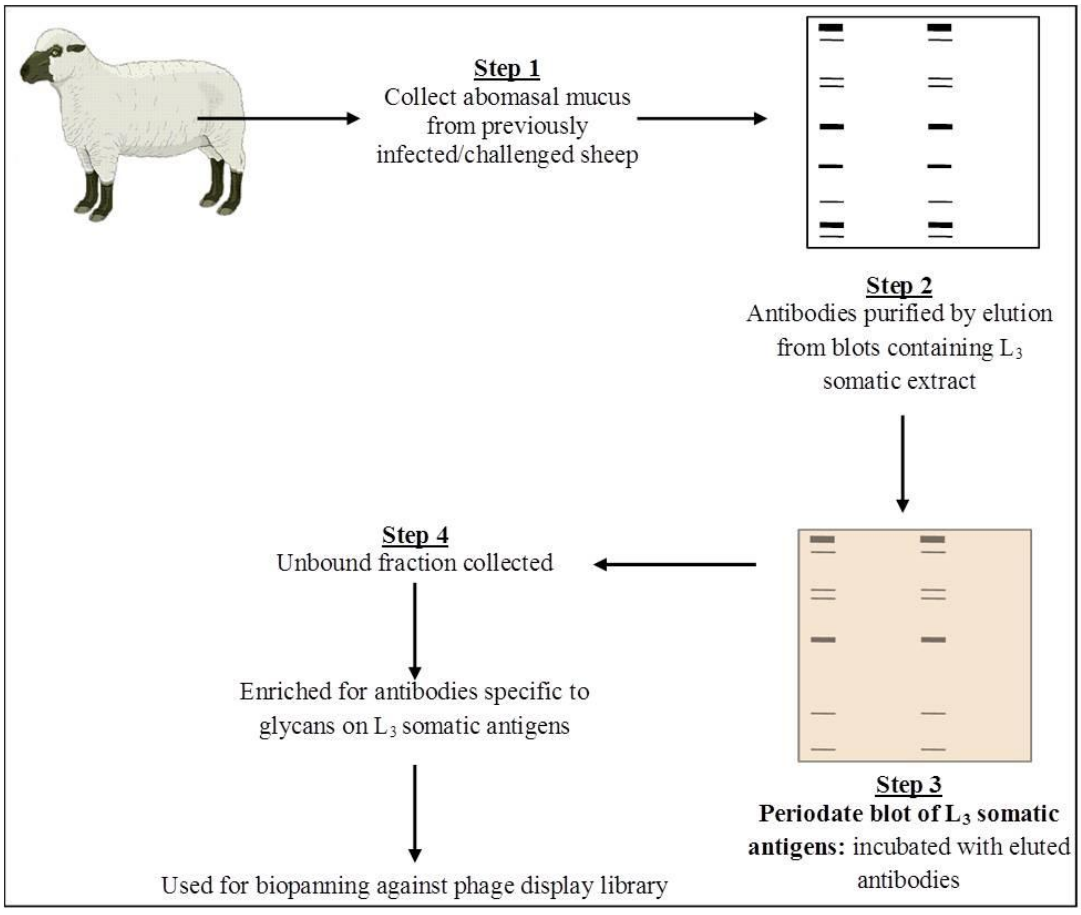

Figure 4.2 Schematic illustration of process for purification of abomasal mucus antibodies against glycans on *T. circumcincta* L_3 somatic antigens.

The unbound fraction was collected. IgA reactivity of antibodies in this unbound fraction to glycans present on *T. circumcincta* L_3 somatic antigens was confirmed by immunoblotting of *T. circumcincta* L_3 somatic extract, using anti-ovine IgA conjugate as a secondary antibody (section 2.2.7).

4.2.2 Biopanning of purified abomasal mucosal antibodies against a 7-mer phage display library

Abomasal mucosal antibodies purified against glycans on *T. circumcincta* L_3 somatic antigens were used for screening commercially available heptapeptide (PhD-7™) M13 pIII libraries (New England Biolabs Inc, USA). The phage display kit was used according to the manufacturer's instructions. Briefly, purified abomasal mucus antibodies (see section 4.2.1) were diluted in coating buffer (0.1M Na_2CO_3, $NaHCO_3$; pH 9.6) to 10µg/ml, and used to coat a 90 mm Petri dish overnight at 4°C. The dish was washed six times with TBST [50mM Tris-HCl (pH 7.5), 150mM sodium chloride, 0.1% (v/v) Tween-20]. Following washing, plates were incubated with a blocking buffer [0.1M sodium carbonate, 5 mg/ml bovine-serum albumin (BSA), pH 8.6] for 2 h at 4°C to prevent non-specific binding. Plates were incubated with a 1:100 dilution (in TBST) of the 1 x 10^{11} phage library for 1 h at room temperature. Unbound phage were removed by washing with TBST; high-affinity bound phage were eluted with 1 ml of the elution buffer (0.2M glycine-HCl, 1mg/ml BSA, pH 2.2) and neutralised with 150 µl/ml of 1M Tris-HCl (pH 9.1). This eluted phage was amplified, as described below, prior to use as the input phage for the subsequent rounds of panning with three complete rounds of panning conducted in total.

4.2.2.1 Amplification and titration of phage clones selected through biopanning with purified abomasal mucosal antibodies

Phage eluates were amplified in *Escherichia coli* strain K12 ER2738 following the 1st and 2nd rounds of biopanning against purified antibodies. Phage eluted from the biopanning round were added to a 20 ml K12 ER2738 Luria-Bertani (LB) broth

(Appendix 1) culture in early log-phase of growth and were incubated for 4.5 h at 37°C with shaking at 200 rpm. The culture was centrifuged for 10 min at 12000 *g* at 4°C to pellet *E. coli* cells. The supernatant was transferred to a 50 ml tube, and 1/6th of the volume of 20% polyethylene glycol-8000 (PEG-8000)/2.5M NaCl added to aid precipitation of phage from cell supernatants. Phage were precipitated overnight at 4°C. The PEG/phage precipitation was centrifuged at 12000 *g* for 15 min at 4°C and the supernatant discarded. Phage formed a white fingerprint-sized smear on the side of the tube and were resuspended in 1 ml of TBS. The suspension was transferred to a 1.5 ml tube and centrifuged at 9000 *g* for 5 min at 4°C to pellet residual cells. The supernatant was transferred to a fresh 1.5 ml tube, re-precipitated by the addition of 1/6th volume of 20% PEG-8000/2.5M NaCl and incubated on ice for 1 h. The phage/PEG suspension was centrifuged again at 9000 *g* for 10 min at 4°C, the supernatant discarded and the phage pellet resuspended in 200 µl of TBS. This was termed 'the amplified eluate' and was stored at 4°C until required for the input phage step in the subsequent panning round. The 'plaque-forming units' (PFU) per ml of the amplified phage eluates were estimated through titration as follows. For titration of unamplified panning eluates and amplified phage culture supernatants, 10 ml of LB broth with ER2738 were inoculated with a loopful of ER2738 from the LB agar/Tet (Tetracycline, 20 mg/ml) stock plate and then incubated at 37°C for 3.5 h with shaking at 200 rpm. Whilst the cells were growing, Top Agar (see Appendix 1 for recipe) was melted and 3 ml dispensed into sterile culture tubes, one per phage dilution and kept at 45°C until required. One LB/IPTG (isopropyl-β-D-thiogalactoside; Promega)/X-gal(5-Bromo-4-chloro-3-indolyl-β-D-galactoside; Promega) plate per dilution (see Appendix 1 for recipe) was pre-warmed for 2 h at 37°C until required. Phage eluates were serially diluted 10-fold in LB broth, in the following dilution ranges: unamplified panning eluates 10^{1}–10^{4}; amplified phage culture supernatants 10^{6}–10^{10}. To carry out the infection of *E. coli* with phage, 200 µl of mid-log phase ER2738 were dispensed into 1.5 ml tubes, 10 µl of the appropriate phage dilution were added, vortexed and incubated at room temperature for 5 min. The infected cells were transferred, one infection at a time, to culture tubes containing 45°C

Top Agar, vortexed briefly and the culture immediately poured onto a pre-warmed LB/ITPG/Xgal plate. The plates were allowed to cool for 5 min, inverted and incubated overnight at 37°C. After 16 h, blue plaques were counted on those plates that had approximately 100 plaques and multiplied by the dilution factor to give the phage titre in plaque-forming units (pfu) per 10 µl. The cloning vector used in the generation of the phage display library is derived from the common cloning vector, M13mp19, which contains the lacZα gene and the M13 phage plaques appear blue when plated on LB media containing X-gal and IPTG. Environmental filamentous phage appeared as white plaques on the media and were not selected. Phage clones selected in this way were then used for DNA sequencing and target specificity confirmation by phage ELISA. Fifty clones were selected and isolated for initial DNA sequencing. Subsequently, 20 of the sequenced clones also had a phage stock prepared for the assessment of target specificity by ELISA. In both cases it was necessary to re-amplify phage from individual plaques to obtain sufficient quantities to work with. For this, an overnight culture of ER2738 was diluted 1:100 in LB, then 1 ml was dispensed into sterile culture tubes, one for each clone to be characterised. A sterile pipette tip was used to stab a suitably discrete blue plaque from the round three titration plates and transferred to a tube containing the diluted bacterial culture. Transfected cultures were incubated at 37°C for 5 h, shaking at 200 rpm. Cultures were centrifuged at 9000 *g* for 1 min to pellet bacterial cells, the supernatant was retained and was classified as the "amplified phage stock" and was stored at -20°C diluted 1:1 with sterile glycerol.

4.2.2.2 Biopanning with "control" antibodies from sheep abomasal mucus

A control experiment was performed to address the potential issue of isolating phage clones that had been selected through non-specific interactions with antibodies or host proteins present in the abomasal mucus antibody preparations. Biopanning experiments were conducted with purified antibodies from abomasal mucus collected from sheep given a single infection (termed "M-CO", see Table 4.1). The protocol followed for biopanning was the same as detailed in Section 4.2.2 with the following modification:

in the coating steps, Petri dishes were coated with 10 µg/ml of abomasal mucus antibodies collected from sheep infected with a single bolus challenge infection of 50,000 *T. circumcincta* L_3 (termed 'M-CO' and detailed in section 2.2.1.1).

4.2.2.3 Preparation of phage DNA sequencing templates

For preparation of phage DNA sequencing templates, 500 µl of phage-containing supernatant generated as described in sections 4.2.2.1 and 4.2.2.2 were added to 200 µl of 20% PEG-8000/2.5M NaCl for 20 min at room temperature to precipitate phage. The tubes were centrifuged at 9000 *g* for 10 min at 4°C and the supernatant discarded. Phage pellets were resuspended in 100 µl of iodide buffer (Appendix 1). Single-stranded phage DNA was then precipitated by the addition of 250 µl of 100% ethanol to the samples and incubation for 20 min at room temperature. The phage DNA preparations were centrifuged at 9000 *g* for 10 min at 4°C and the supernatant discarded. Phage DNA pellets were washed with 500 µl of 70% ethanol (at -20°C), re-centrifuged at 9000 *g* for 10 min at 4°C and the pellet air-dried. Phage DNA pellets were resuspended in 30 µl of molecular biology grade water and stored at -20°C. Phage DNA was quantified by spectrophotometry (Nanodrop ND-1000 UV-Vis Spectrophotomer) and 20 µl of each phage clone were sent for DNA sequencing at Eurofins MWG with the 96gIII primer (5' CAACAAATCGTTTTAGGGTATG 3').

4.2.3 Sequence analysis of immunoreactive heptapeptides from a phage-display library

Sequences were assembled and the specific locations of sections of the M13KE vector were located prior to isolation of the insert sequence representing the heptamer. Briefly, as sequencing was on the anti-sense strand of the M13KE vector, the reverse complement sequence was derived from the raw data. Specific sections of the M13KE vector sequence were searched for (Figure 4.3). This was to ensure that the DNA sequence obtained matched that of the M13KE vector and the randomized region

contained a single sequenced insert. Also, the third nucleotide of each codon within the heptamer in the randomized region was confirmed to be either G or T and the Gly-Gly-Gly leader sequence located (Figure 4.3). These sequence checks facilitated identification of the location of the insert sequence. The insert sequences were translated to obtain the peptide sequence displayed by the phage clone.

GTAAATGAATTTTCTGTATGGGATTTTGCTAAACAACTTTCAACAGTTTCGGCCG
A**ACCTCCACC**CTGCGGCTTCGAAGAATTCATAGAGTGAGAATAGAAAGGTACC
ACTAAAGGAATTGCGAATAATAATTTTTTCACGTTGAAAATCTCCAAAAAAAAG
GCTCCAAAAGGAGCCTTTAATTGTATCGGTTT

Figure 4.3 Example of a section of the M13KE vector DNA sequence obtained through sequencing of phage clones isolated by biopanning purified ovine abomasal mucosal antibodies against a 7-mer phage display library.

The sequence obtained corresponds to the anti-sense strand (5' to 3') of the M13KE vector, and the following areas of the vector sequence were located prior to isolation of the randomised region:

(i) Hybridisation site of -28gIII primer (highlighted in grey)
(ii) Flanking region of the library insert incorporating *Acc*651 restriction site (highlighted in pink) indicates start of insert sequence
(iii) Flanking end region of the library insert incorporating *Eag*I restriction site (highlighted in green) indicates end of insert sequence
(iv) Randomised region (highlighted in yellow)
(v) Gly-Gly-Gly leader sequence of DNA insert (underlined in yellow region)

4.2.4 Bioinformatic analysis of peptide sequences

Peptide sequences obtained through DNA sequencing of isolated phage clones were analysed to assess if any of the identified sequences were classified as propagation target unrelated peptides (TUPs) through the depository database MimoDB version 3 (http://www.immunet.cn/mimodb). Propagation TUPs are phage clones that have a distinct growth advantage over other phage clones in the library, often due to a mutation. As a result they are enriched during the amplification and propagation steps following phage selection (Thomas, 2010; Vodnik, 2011). To identify if any peptide sequences shared or matched linear peptide sequences already associated with previously identified parasite proteins, the peptide sequences were also BLAST (Basic Local Alignment Search Tool, Altschul *et al.*, 1990) searched against the publically available databases,

National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov) and WormBase 2 WS234 (http//www.wormbase.org/#012-3-6).

4.2.5 Assessment of specificity of phage clones for target antibodies by ELISA

Target specificities of selected phage clones were assessed by distinguishing true target binding from background binding to both the plastic support and the BSA blocking agent. This was achieved through comparison of the level of binding in uncoated wells of the 96-well plate to levels in wells coated with the target IgA (as illustrated in Figure 4.4). To prepare phage clones for assessment by ELISA, amplified phage stocks of 20 clones (see section 4.2.2.1) were re-amplified to obtain sufficient quantities for the assays. Briefly, overnight cultures of ER2738 in LB were diluted 1:100 in 20 ml of fresh LB, with one new culture for each clone to be characterized. Phage glycerol stocks (5 µl each) were added to a 20 ml culture and incubated at 200 rpm, 37°C for 5 h. The culture was centrifuged at 12000 *g* for 10 min at 4°C and the supernatant transferred to a 50 ml tube. To the phage containing supernatant, 1/6th of the volume of 20% PEG-800/2.5M NaCl was added and the phage were precipitated at 4°C overnight. The PEG precipitations were centrifuged at 12000 *g* for 15 min at 4°C and the supernatant discarded. The phage pellet was resuspended in 1 ml of TBS, transferred to a 1.5 ml tube and centrifuged at 9000 *g* for 5 min at 4°C to pellet residual cells. The supernatant was transferred to a fresh 1.5 ml tube, re-precipitated with 1/6th volume of 20% PEG-800/2.5M NaCl and incubated for 1 h on ice. The phage precipitation solutions were centrifuged at 9000 *g* for 10 min at 4°C and the supernatant discarded. The amplified phage clone pellet was resuspended in 50 µl of TBS and stored with an equal volume of sterile glycerol at -20°C. For each phage clone, three wells of a 96-well plate were coated with 100 µl of L_3-purified antibody (section 4.2.1) at 100 µg/ml in 0.1M sodium carbonate, pH 8.6. Three wells of the 96-well plate were left uncoated for each clone (Figure 4.4) and the plates incubated overnight at 4°C. Excess target (antibody) solution was removed and the plates washed six times with TBST. Plates were blocked for non-specific interactions with blocking buffer [TBST + 5% (w/v) BSA] at 200µl/well for 2 h

at 4°C. The uncoated wells were also blocked to test for binding of each selected peptide sequence to BSA-coated plastic. Blocking buffer was aspirated and the plates washed six times with TBST. Phage clones were diluted 1:100 from the stock (10^{14} pfu/ml) and 100 µl of this preparation added to each well of the assay plate followed by incubation at room temperature for 2 h on an orbital shaker. After six washes with TBST, an anti-M13 mouse monoclonal HRP-conjugated antibody (GE Healthcare, #27-9421-01) was diluted 1:1000 in TBST and 100 µl of this added to each well, and incubated at room temperature for 1 h.

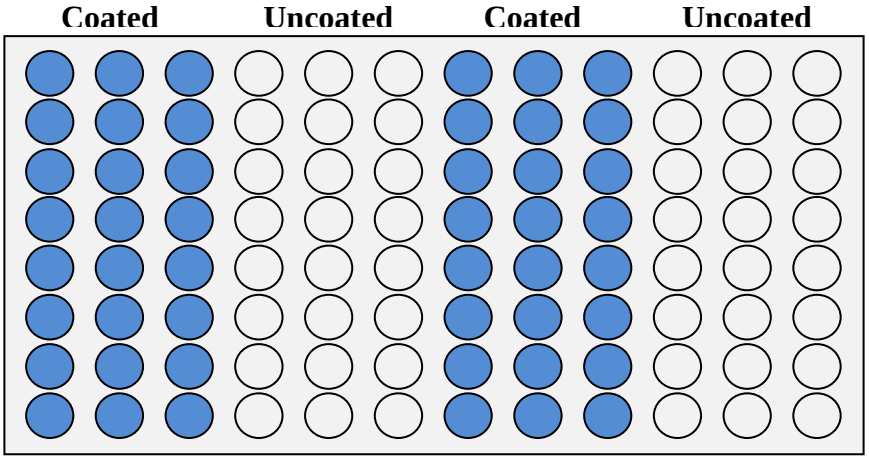

Figure 4.4 Schematic illustration of the plate template for the ELISAs used to assess and compare the background binding of the phage clones under investigation to the specificity for the target antibodies.

One row of the plate was used for each phage clone. For each phage clone under investigation, three wells were coated with the target antibody used in the biopanning, L_3-purified IgA (100µl/well) (blue wells). Three wells were uncoated and only received the BSA-blocking buffer (clear wells).

After final washes in TBST, OPD (50 µl/well) was applied and the plates incubated at room temperature in the dark for 20 min. The reaction was stopped by the addition of 25 µl 2.5M H_2SO_4 and the absorbance measured at 490nm. For each phage clone, the OD values obtained from the wells coated and uncoated with the target were compared.

4.2.6 Immunoblot of phage clones to detect the binding of abomasal mucosal IgA

Five microlitres of the phage supernatant (as prepared in Section 4.2.2.1) of four phage clones (Clone 2: LPLTPLP, Clone 6: HAIYPRH, Clone 8: WPTLQWA, Clone 20: YGFVPSW) were pipetted onto an 8cm x 8cm square of 0.45 µm pore-size nitrocellulose membrane (cut into 5 strips) and left to air dry at room temperature for 2 h. As a positive control, 2 µg *T. circumcincta* L_3 somatic extract (section 2.2.4) were also applied to the membrane. Five microlitres of an overnight culture of ER2738 were also added to assess the presence of any background reactivity of ovine antibodies to the bacterial strain used for the propagation of phage. Once dry, the strips were blocked against non-specific interactions by incubation in TNTT overnight at 4°C. Strips were incubated with primary antibody (abomasal mucus from M-PI or M-HF [helminth-free], Table 4.1), diluted 1:5 in 5 ml of TNTT, and were incubated for 2 h at room temperature as follows: three strips were probed with abomasal mucus from individual M-PI sheep, one strip was incubated with TNTT as the "no primary antibody" control and one strip was incubated with a pool of abomasal mucus from M-HF sheep. After the primary antibody incubations, strips were washed three times in TNTT (10 min/wash) and incubated in mouse monoclonal anti-ovine/bovine IgA (Serotec, MCA628) (diluted at 1:250 in 5 ml of TNTT) for 1 h at room temperature. Following re-washing in TNTT, strips were incubated in polyclonal rabbit anti-mouse immunoglobulins-HRP conjugate (Dako, P0260) (diluted at 1:1000 in 5 ml of TNTT) for 1 h at room temperature. Following the final washes, detection was carried out using DAB (3,3–Diaminobenzidine) and stopped after 6 min with a rinse in distilled water.

4.2.7 Optimisation of ELISA to assess immunoreactivity of selected peptides

Several ELISA methods were used to detect and quantify the level of ovine IgA in abomasal mucus and efferent gastric lymph from M-PI and G-PI sheep (section 2.2.1.1)

that bound to the selected phage clones. Due to time and material constraints only two clones (8 and 10) were tested in the immunoreactivity investigations.

a) Direct phage ELISA

Phage clones, diluted in coating buffer, were coated directly onto the surface of the polystyrene wells. To optimize this, checkerboard titration ELISAs of the phage dilution (10^{14}–10^{1} pfu/ml), primary antibody dilution (1:20–1:500) and conjugated secondary antibodies (1:250–1:4000) were investigated. Following this optimisation, the phage clones, at a concentration of 10^{12} pfu/ml (50µl/well) in phage coating buffer (0.1M $NaHCO_3$, pH 8.6), were coated to the surface of the plates and incubated overnight at 4°C. Plates were then washed six times with PBST and non-specific binding blocked as described in Section 4.2.7 (a). After re-washing, plates were incubated with 50 µl of primary antibody (the samples were the same as used in section 4.2.7 (a) and were diluted 1:20 in PBST) and incubated for 2 h at 37°C. Plates were re-washed in PBST and the subsequent secondary antibody incubation and detection of binding performed as detailed in section 4.2.7 (a). All primary antibody samples were in duplicate on each plate and each plate was repeated in triplicate. The absorbance values were collated from the six independent readings across the three plates and presented as the mean with the standard deviation.

b) Synthetic peptide ELISA:

Two biotinylated peptides, representing the heptamer sequences of two immunoreactive phage clones from the library screen, (HAIYPRH = equivalent to clone 2 and WPTLQWA = equivalent to clone 8) were synthesized by Mimotopes Pty Ltd, UK (see Figure 4.5).

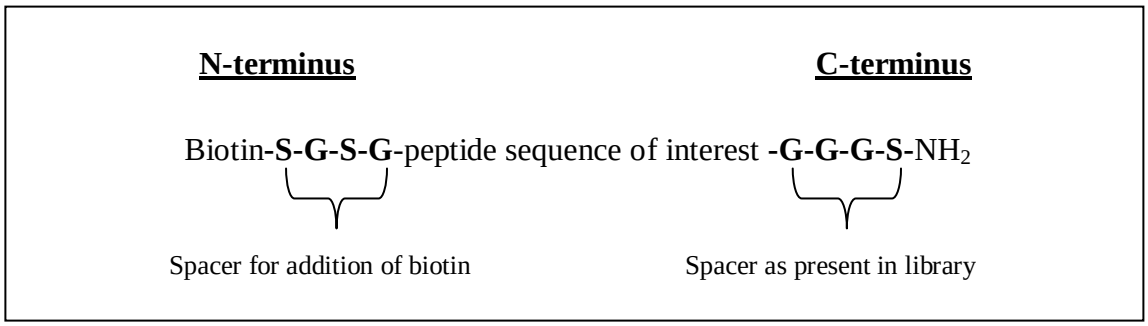

Figure 4.5 Schematic illustration of the modifications to the design of the synthetic peptides, which were based on the 7-mer peptide sequences, identified through biopanning purified abomasal mucosal antibodies with a phage display library.

The modifications in the design of the synthetic peptides were as follows:

(i) Amidation of C-terminal carboxylate to block the negative charge of the free carboxyl group. During panning the C-terminus of the inserted sequence is fused to phage so does not have a free negatively charged carboxylate and if left unmodified it would abolish binding.

(ii) A Ser-Gly-Gly-Gly peptide spacer inserted between the random sequence and biotin tag .

(iii) A Gly-Gly-Gly-Ser peptide space between the C-terminus end of the random sequence and pIII sequence.

To provide a positive control, *T. circumcincta* L_4 ES products (prepared as described in Section 2.2.3) were biotinylated with a commercially available Biotin labeling kit (Abnova, US, KA0003), according to the manufacturer's instructions. *T. circumcincta* L_4 ES products were used because of the limited availability of *T. circumcincta* L_3 somatic extract at the time the assay was conducted. Pre-BSA blocked streptavidin-coated clear 96-well plates (Thermo Scientific, 15500) were washed four times with PBST. The stock solution of each synthetic peptide [1 mg/ml in DMSO (dimethyl sulphoxide; Sigma-Aldrich)] was diluted 1:1000 in PBST and 100 µl were applied into the corresponding well positions (in triplicate). Plates were incubated for 1 h at room temperature. Plates were re-washed in PBST before primary antibody incubations. Plates incubated with 100 µl of primary antibody (the samples were the same as used in section 4.2.7 (a) and were diluted 1:500 in PBST) and incubated for 1 h at room temperature. Plates were re-washed in PBST and the subsequent secondary antibody incubation and detection of binding was as detailed in section 4.2.7 (a).

4.2.8 Definitive phage ELISAs probed with efferent gastric lymph IgA from sheep with differing levels of acquired immunity to *T. circumcincta*

Four phage clones (Clone 2, LPLTPLP; Clone 6, HAIYPRH; Clone 8, WPTLQWA; Clone 10, YGFVPSW) were investigated to quantify binding of IgA in efferent gastric lymph from previously infected/challenged sheep to the structures presented by the peptide sequences. Further analysis was conducted to identify if binding to the peptide clones was related to known correlates of immunity in these animals: worm burden, percentage of inhibited *T. circumcincta* L_4 and total concentration of IgA in efferent gastric lymph at 7 dpc. Following optimisation (section 4.2.7), the "direct phage ELISA" was employed: microtitre plates were coated with an individual phage clone diluted to 10^{12} pfu/ml in coating buffer (50µl/well) overnight at 4°C. Following washes in PBST, primary antibody incubations were conducted at room temperature for 1 h. The source of primary antibodies was individual efferent gastric lymph samples collected at a single time-point during the experimental infection: "G-PI" at 7 dpc, "GL-CO" at 7 dpc and "GL-HF" at 0 dpc (see Table 4.1). All remaining procedures were as documented in Section 4.2.7 (b).

4.2.9 Statistical analysis

Data obtained from both assays investigating the level of ovine IgA binding to the two phage clones/peptide structures and the relationships between the level of IgA binding and immune parameters differed significantly from a normal distribution (Anderson Darling Test, $p<0.05$). The median values obtained in the ELISAs investigating the level of ovine IgA binding to the two phage clones/peptide structures from the different infection groups were statistically evaluated by the non-parametric Friedman two-way ANOVA test. An interactions analysis was used to analyse interactions between parasite challenge status, clone identity and inter-plate variability between plates.

This was conducted through a univariate Generalised Linear Model (GLM), with the following formula:

$$OD \sim (Plate) + Challenge + Clone + Challenge*Clone$$

The formula was adjusted to take into account plate-to-plate variance, represented by '(Plate)'. It also took into account the effect of two independent variables on the OD value obtained from the ELISA. This was shown by 'Challenge' representing the infection status (previously infected/challenged or helminth-naïve) and 'Clone' which represented the phage clone under investigation. The benefit of a GLM is that it also investigates the interactions between mutilple factors/variable and their impact upon the OD, this was incorporated into the formula as indicated by 'Challenge*Clone'.

The relationships between the levels of IgA binding to the selected peptide sequences and the immune parameters were summarised by non-parametric correlation analysis using Spearman rank correlation coefficient (SPSS version 19). For all statistical analysis tests, the level of significance was set at $p<0.05$.

4.3 Results

4.3.1 Affinity purification of ovine antibodies which bind glycans on *T. circumcincta* L_3 somatic antigens

Following the double immunoblot purification procedure (Figure 4.2) for purifying antibodies that bound structural glycans, the unbound fraction (containing the purified antibodies) was retained. The fraction that did not bind the periodate-treated blot of *T. circumcincta* L_3 somatic extract was examined to establish whether the antibodies present were able to bind to L_3 somatic antigens (Figure 4.6).

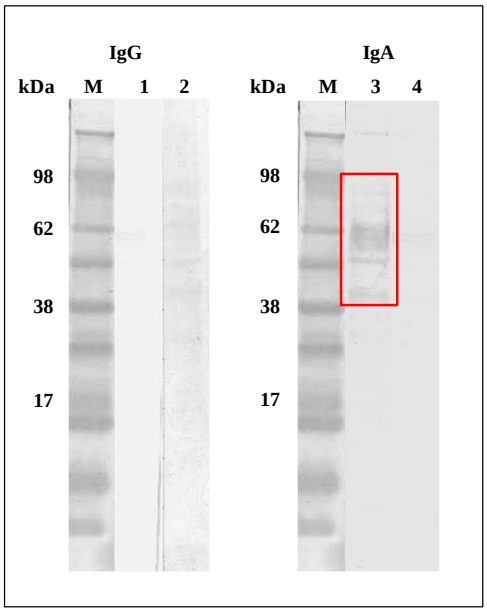

Figure 4.6 Immunoblot of *T. circumcincta* L_3 somatic extract probed with purified (antibodies were pre-purified against *T. circumcincta* L_3 somatic antigens) antibodies from the abomasal mucus obtained from previously infected/challenged sheep.

Lanes 2 and 3: probed with abomasal antibodies purified against glycans on *T. circumcincta* L_3 somatic antigens through a double immunoblot purification procedure. Lanes 1 and 4: no primary antibody controls. All lanes were incubated with the appropriate secondary and tertiary antibodies for the isotype under investigation (lanes 1 and 2, IgG; lanes 3 and 4, IgA) and developed with SigmaFast DAB™. Lane M. represents molecular weight markers given in kDa. The red box surrounds the area of IgA reactivity (50-200kDa) in abomasal mucus of previously infected/challenged sheep after purification against *T. circumcincta* L_3 somatic antigens.

Purified abomasal antibodies bound antigens present in *T. circumcincta* L_3 somatic extracts (Figure 4.6). Numerous IgA-reactive bands were detected between 50 and 200 kDa (Figure 4.6, Lane 3). Little IgG reactivity was observed against *T. circumcincta* L_3 somatic antigen extracts in this fraction (Figure 4.6, Lane 2). This purified, IgA-enriched antibody preparation was used for biopanning the 7-mer phage display library to identify peptides sequences that potentially mimic the parasite's structural epitopes.

4.3.2 Biopanning of purified IgA against a 7-mer phage display library

To determine phage enrichment after each round of panning the heptamer phage library with L_3 antigen- reactive IgA, the titre of the amplified phage eluted was determined and quantified as plaque-forming units per ml (pfu/ml). The amplified phage eluate from the first and second rounds of panning increased from 1.74×10^8 plaque pfu/ml to 1.56×10^{10} pfu/ml (Table 4.2). At the end of round three, the eluted phage titre was 1.31×10^6 (Table 4.2).

Round	Input phage (pfu/ml)	Amplified phage eluted (pfu/ml)
1	1.0×10^{11}	1.74×10^8
2	1.0×10^{11}	1.56×10^{10}
3	1.0×10^{11}	1.31×10^{6}*

Table 4.2 Titres of input phage and the amplified phage eluted from each round of biopanning a heptamer phage display library with abomasal IgA purified against *T. circumcincta* L_3 glycans. * denotes unamplified eluate. Pfu/ml = plaque-forming units per ml.

Following three rounds of screening, 50 phage clones with affinity for the purified IgA used in the panning experiments were selected at random from agar plates (Section 4.2.2.1), phage DNA prepared and sequenced to enable the peptide sequences of the insert regions to be determined (Appendix 3 provides full list of translated sequences). Peptide sequences that were repeated (> 2 times) are shown in Table 4.3. Bioinformatic analysis of these sequences revealed that two, HAIYPRH and LPLTPLP, were classified as "propagation target-unrelated peptides (TUPs)" and have been identified in previous studies (Brammer *et al*., 2008; Huang *et al*., 2011; Ru *et al*., 2010; Vodnik, 2011). These are phage clones that have a distinct growth advantage over other clones and as a

result are enriched during the amplification and propagation steps following phage selection. This means that while they may be specifically bound by antibody in the panning stage, they are likely to be over-represented at the sequencing stage. In the complete dataset, seven further TUPs were identified (Appendix 3); however, they were not as abundant as the two detailed above. The control experiments using abomasal mucus from sheep exposed to a single challenge of *T. circumcincta* ("M-CO", Table 4.1) resulted in the selection of three clones which also bound affinity-purified M-PI antibodies: HAIYPRH, KLPGWSG and YSIPKSS (Table 4.3 and Appendix 3).

Peptide	**Number of repetitions**	**Target-unrelated peptide**	**Identified by other studies***	**Identified by "Control" antibodies**	**Clone ID assigned for further experiments**
AKIDART	4				1
LPLTPLP	2	Yes	Yes		2
SAPSSKN	2				3
FMRSPPM	2				4
SPSMLQK	2				5
HAIYPRH	9	Yes	Yes	Yes	6
KLPGWSG	2			Yes	7
WPTLQWA	10				8
GSHNPHL	2				9
YGFVPSW	3				10

Table 4.3 Frequency of repeated peptide sequences from phage clones.

Clones were selected through panning a 7-mer phage display library with IgA purified against glycans on *T. circumcincta* L_3 somatic antigens. * Listed on the MimoDB search facility compiled of collated data from phage display library panning experiments.

Analysis of the proportion of different classes of amino acids in the peptide sequences revealed that there were high proportions of amino acid residues belonging to the aromatic, hydrophobic and basic groups (Figure 4.7).

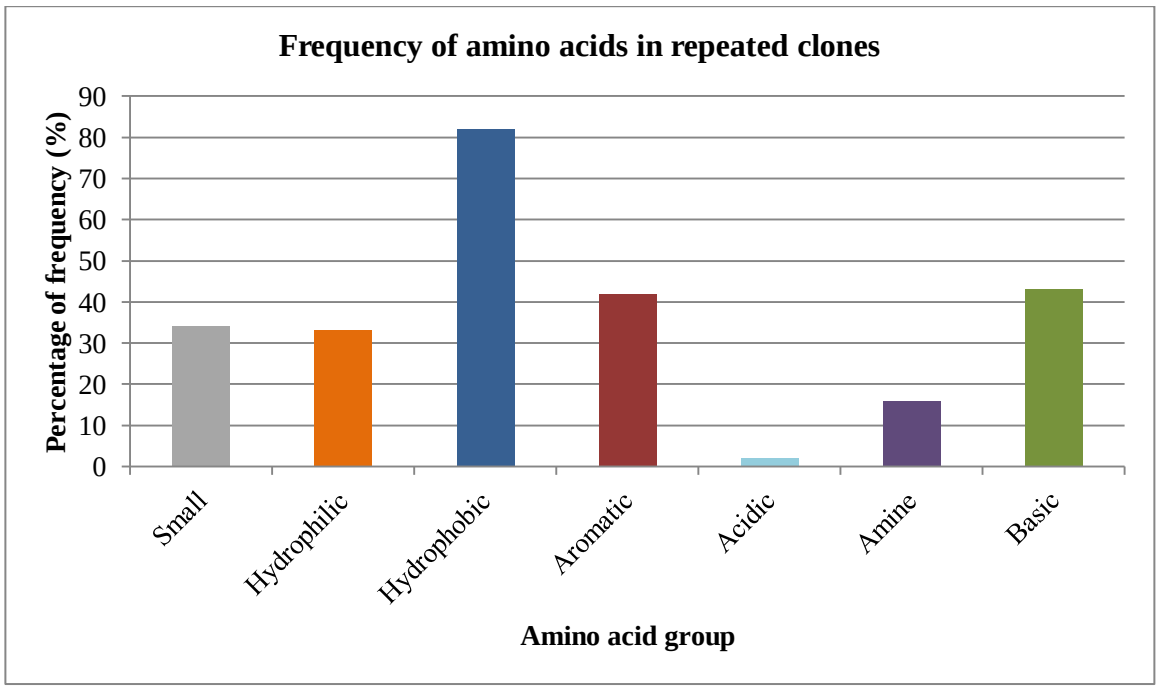

Figure 4.7 Frequency of amino acid groups in peptide sequences identified via biopanning a random heptamer phage display library with *T. circumcincta* L_3 antigen selected IgA.
Groups of amino acids:
Small (Glycine, Alanine); Hydrophilic (Serine, Threonine, Cysteine); Hydrophobic (Valine, Leucine, Isoleucine, Methionine, Proline); Aromatic (Phenylalanie, Tyrosine, Tyrptophan); Acidic (Aspartic acid, Glutamic acid); Amine (Asparagine, Glutamine); Basic (Histidine, Lysine, Arginine).
Percentage frequency of amino acid groups was calculated by assessing the total number of repetitions of individual amino acids in the sequence dataset as a proportion of the total number of amino acids.

4.3.3 Binding of phage-displayed heptamer peptides by abomasal mucosal antibodies

Twenty of the phage clones selected above were evaluated for immunoreactivity by ELISA by comparing the level of background (i.e. binding to uncoated wells) to the level of binding to purified IgA coated wells (Figure 4.8). The antigen-specific IgA bound all twenty phage clones. Clones 1, 2, 4, 6, 8 and 10 demonstrated the highest levels of binding (Figure 4.8). Clones 2 and 6 bound IgA in the abomasal mucus of previously infected/challenged M-PI sheep (Figure 4.8): both clones are TUPs and clone 6 was also bound by IgA from sheep exposed to a single challenge. Thus, with the exception of using these clones as positive controls in immunoblotting and synthetic

peptide binding experiments (Clone 6, Figures 4.9 and 4.12), these clones were not analysed further.

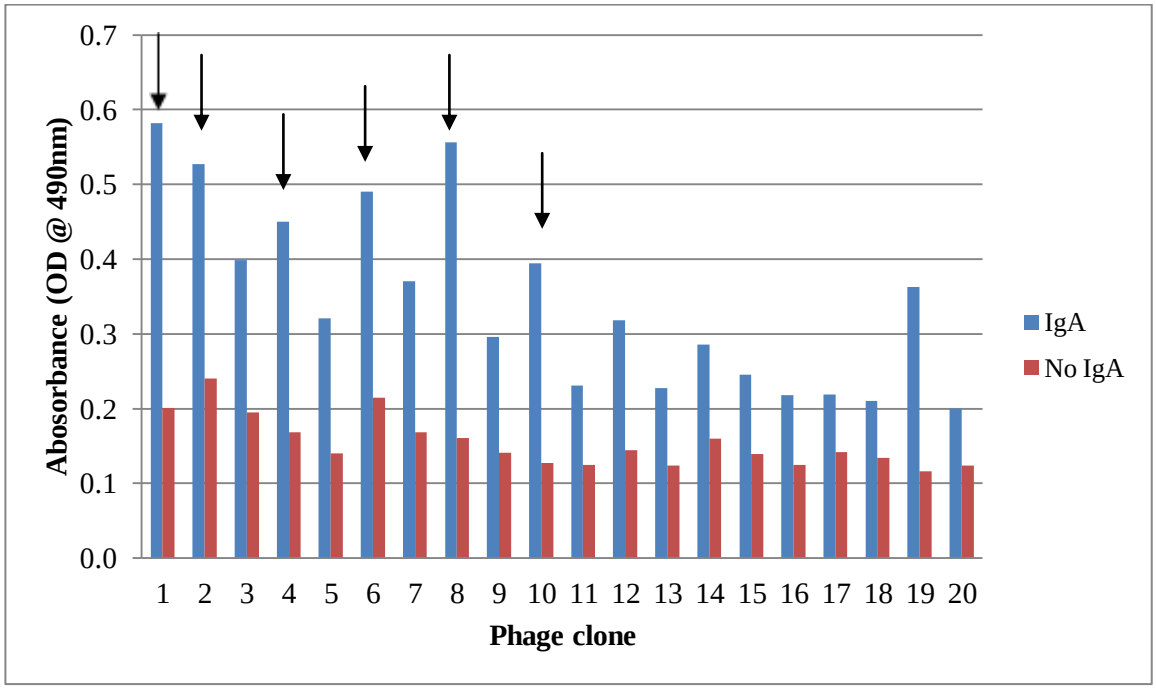

Figure 4.8 Screening ELISA used to investigate target specificity of twenty selected phage clones by comparison of the level of specific binding to IgA coated plates versus uncoated plates.
Arrows denote clones selected for further investigation. Clones 1, 2, 4, 6, 8 and 10 gave the highest OD values in the ELISA when the mean levels of binding in IgA-coated wells (blue bars) were compared to the uncoated wells (red bars).

4.3.4 Immunoreactivity of selected peptide sequences through dot-blots probed with abomasal mucus

An immunodotblot was used to investigate if IgA in abomasal mucus from sheep experimentally infected with *T. circumcincta* L_3, through a trickle infection/challenge regime, bound to the peptide structures displayed by the phage clones (Figure 4.9). All phage clones bound mucosal IgA from previously infected/challenged sheep (termed "M-PI", as described in section 2.2.1.1) (Figure 4.9, Lanes 1-3). IgA binding was not observed in the lane containing phage clones probed with abomasal mucus from

helminth-naïve sheep (Figure 4.9, Lane 4). No binding of mucus IgA from previously infected/challenged or helminth-naïve sheep (Table 4.1) to the bacterial expression strain used in the construction of the phage display library was evident (Figure 4.9, Lanes 1-4).

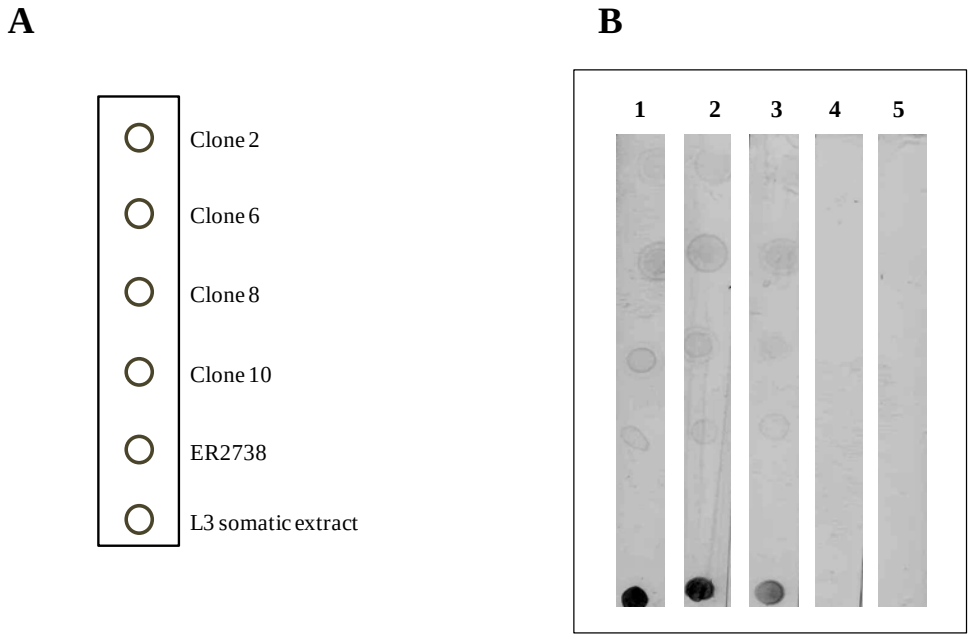

Figure 4.9 Immuno-dotblot of selected phage clones probed for reactivity to mucus IgA from previously infected sheep.

Four phage clones (5µl) were blotted onto a nitrocellulose membrane. *T. circumcincta* L3 somatic extract (5µg) and *E. coli* ER2738 (5µl) were included as positive and negative controls, respectively. Panel A, schematic illustration of locations of phage clones, *T. circumcincta* L3 somatic extract and *E. coli* ER2738. Panel B, membrane strips probed with samples. Lanes 1–3: Probed with abomasal mucus from previously infected/challenged sheep ("M-PI") (n=3). Lane 4: Probed with a pooled sample of abomasal mucus from helminth-naive sheep ("M-HF") (n=4 in pool). Lane 5: No primary antibody control. Following primary antibody incubations, all lanes were incubated with secondary and tertiary antibodies for detecting IgA binding as described in section 4.2.6).

ELISAs were used to quantify IgA binding. A direct ELISA with phage coated onto the plate for ovine antibody binding and detection was used. Checkerboard titrations of a range of reagents were used to determine the optimal concentrations for the reagents in the assay (data summarised in Table 4.4).

Reagent	Range	Percentage positivity*	OD values	Concentration selected
Polyclonal α-ovine IgA HRP	1:250 to 1:1000	Positive pool: 59% to 7% Negative pool: 18% to 8%	Positive pool: 0.75 to 0.09 Negative pool: 0.24 to 0.10	**1:500**
Efferent gastric lymph (Primary antibody)	1:20 to 1:500	Positive pool: 98% to 46% Negative pool: 17% to 11%	Positive pool: 1.24 to 0.59 Negative pool: 0.22 to 0.15	**1:20**
Phage dilution	10^{14} to 10^{2} pfu/ml	Positive pool: 40% to 6.5% Negative pool: 14% to 5%	Positive pool: 0.318 to 0.083 Negative pool: 0.178 to 0.066	**10^{12} pfu/ml**

Table 4.4 Summarised data from the development and optimisation of reagants and detection antibodies used in the method of the phage ELISA.

*Positive control: *T. circumcincta* L_3 somatic extract (5µg) probed for IgA reactivity with abomasal mucus from previously infected/challenged sheep (termed "M-PI"). Positive pool: Abomasal mucus from previously infected/challenged sheep (termed "M-PI") (n=4). Negative pool: Abomasal mucus from primary infected sheep (termed "M-CO") (n=4).

To maximize available reageants and to minimize background reactivity the following working concentrations and dilutions were used: secondary HRP-conjugated antibody at 1:500, primary antibody at 1:20 and phage dilutions at 10^{12} pfu/ml (Table 4.4). The optimized ELISA was used to quantify binding of clone-specific IgA in abomasal mucus and efferent gastric lymph samples from sheep subjected to a trickle infection. The results are shown in Figures 4.10 and 4.11, for mucus and lymph, respectively.

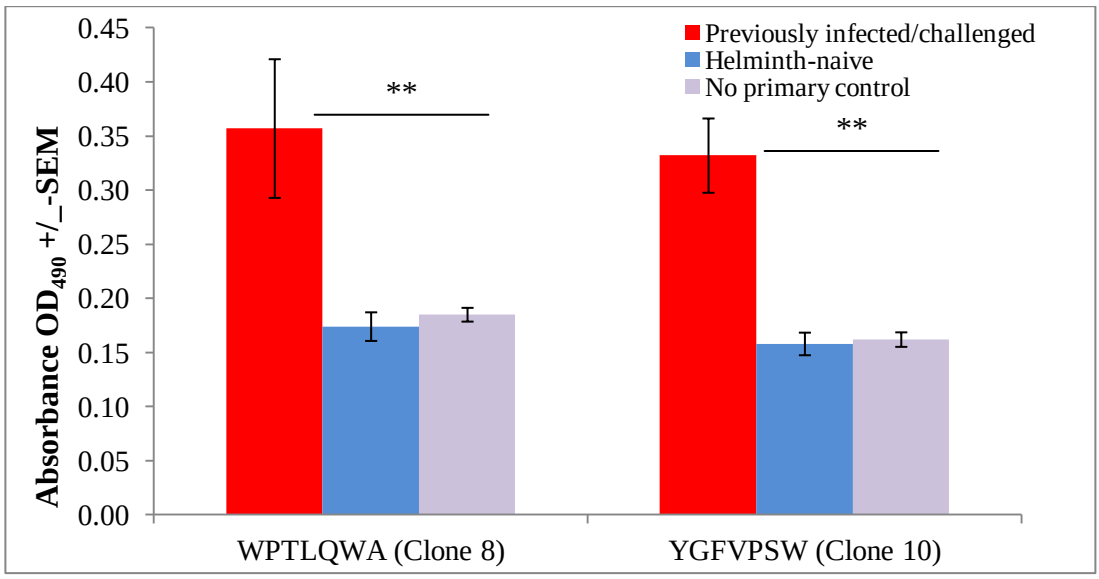

Figure 4.10 Levels of binding of IgA from abomasal mucus to the peptide structures displayed by two phage clones, WPTLQWA (Clone 8) and YGFVPSW (Clone 10), which were selected through panning a phage display library with purified IgA from sheep trickle-infected/challenged with *T. circumcincta* L_3.

ELISA plates were coated with the two phage clones (10^{12} pfu/ml) and incubated with pooled samples of abomasal mucus from groups of sheep. Previously infected/challenged ("M-PI"): abomasal mucus collected from sheep infected with an experimental trickle infection then challenge with 50,000 *T. circumcincta* L_3; the abomasa were collected 2 dpv. Helminth-naive ("M-HF"): Abomasal mucus collected from sheep reared under helminth-free conditions. No primary control: Phage clones probed with developing anti-IgA antibodies only. Results are presented as the median absorbance (n=9) for each group (± SEM). Three independent repetitions of the assay were conducted. '**' denotes statistical significance ($p<0.01$) following comparison of the median OD values between groups.

A generalised linear model (GLM) was used to analyse differences between the groups and the no primary antibody control. This was used to analyse interactions between parasite challenge status, clone identity and inter-plate variability between plates. There was a significant level of inter-plate variability (P=0.012). As a result, the statistical analysis was conducted on the data adjusted for plate variance. The level of binding of IgA from abomasal mucus to the two phage clones was significantly higher (P=0.007) in previously infected/challenged sheep (termed "M-PI") compared to helminth naive sheep (termed "M-HF") and the no primary antibody control (Figure 4.10).

Synthetic peptide versions of two of the heptamer sequences from selected phage clones were also tested in a direct ELISA (Figure 4.11).

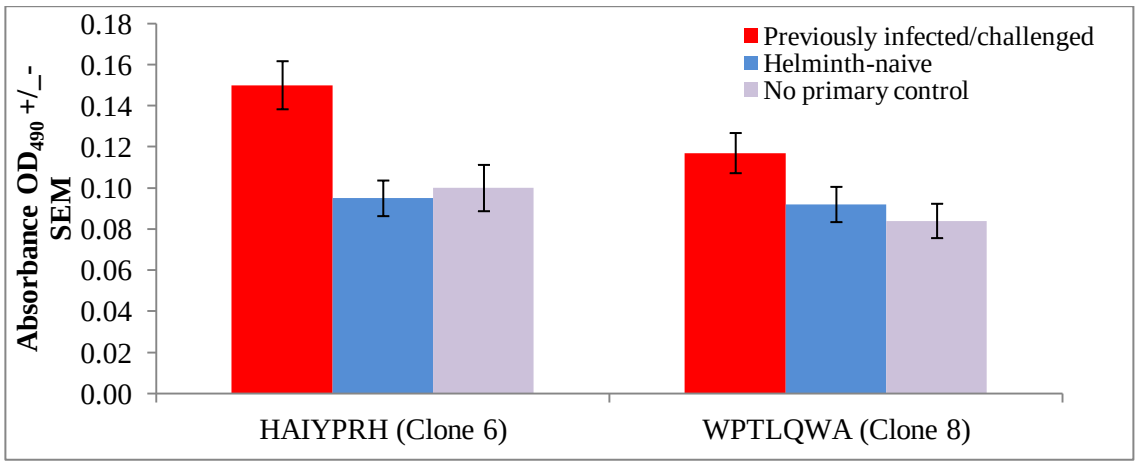

Figure 4.11 Levels of binding of IgA from efferent gastric lymph to two synthetic peptides (HAIYPRH and WPTLQWA) with purified IgA from sheep experimentally trickle-infected/challenged with *T. circumcincta* L_3.

Streptavidin-coated ELISA plates were coated with the biotinylated synthetic peptides at 1:1000 and incubated with pooled samples of efferent gastric lymph from groups. Previously infected/challenged ("G-PI"): pooled efferent gastric lymph collected over 6-10 dpc from previously infected sheep following a bolus challenge of 50,000 *T. circumcincta* L_3. Helminth-naive ("G-HF"): efferent gastric lymph collected from helminth-naive sheep and used as a "negative control". No primary control: Phage probed with anti-IgA developing antibodies only. The sequence of the clones matched up to the following clone numbers: HAIYPRH = Clone 6 and WPTLQWA = Clone 8. The level of IgA binding was detected by HRP-labelled developing antibodies. Results are presented as the mean absorbance (n=6) for each group of sheep (± SEM).

Levels of IgA binding in efferent gastric lymph to peptide sequences in two of the phage clones identified by biopanning were quantified. The samples were obtained from sheep with varying levels of *T. circumcincta* infection after L_3 challenge. Relationships between levels of IgA binding and parasitological and immunological parameters are summarized in Table 4.5.

	Nematode burden		**Total IgA in lymph**		**% inhibited larvae**		**Blast cell response**	
Peptide (Clone)	**R_s value**	**P value**	**R_s value**	**P value**	**R_s value**	**P value**	**R_s value**	**P value**
WPTLQWA (Clone 8)	-0.498	0.030*	0.685	0.001**	0.617	0.005**	0.286	0.493
YGFVPSW (Clone 10)	-0.674	0.002**	0.777	0.000***	0.573	0.010*	-0.238	0.570

Table 4.5 Summary of output from the statistical analysis of relationships between the levels of gastric lymph IgA binding to phage clones 8 and 10 to both the parasitological and immune parameters.

For both phage clones, significant negative correlations (P=0.002 to P=0.03, r_s = -0.498 and -0.674) were observed following comparison of the phage clone-specific IgA responses in efferent gastric lymph samples to the nematode burdens at necropsy (Figure 4.12; Table 4.5).

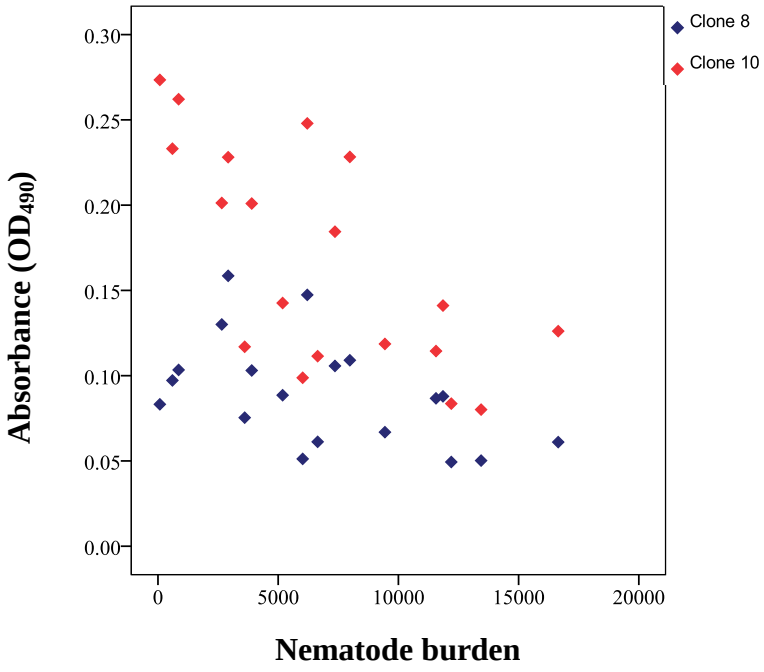

Figure 4.12 Relationship between the level of gastric lymph IgA binding to peptides displayed by phage clones 8 and 10 selected by biopanning and the total nematode burden of sheep previously infected/challenged with *T. circumcincta* L_3**.**

Analysis of the association was conducted using Spearman correlation coefficients. Data points represent absorbance (OD) values from individual gastric lymph samples (n=19). Absorbance values were plotted against the total nematode burden for each individual. The efferent gastric lymph was collected from sheep which had been previously infected with *T. circumcincta* L_3 and subjected to a bolus challenge of 50,000 *T. circumcincta* L_3. The gastric lymph samples used were all from a single time-point (7 dpc). The worm burden of the "GL-PI" sheep was assessed by counting the number of males, females and arrested L4 in a sub-sample of the digests of the abomasal tissue and contents (Halliday *et al*., 2007).

Positive correlations (P<0.001, R_s = 0.685 and 0.777) were found between the level of ovine IgA binding specifically to the peptide structures displayed by the phage clones and the total IgA concentration in the efferent gastric lymph at 7 dpc (Figure 4.13; Table 4.5).

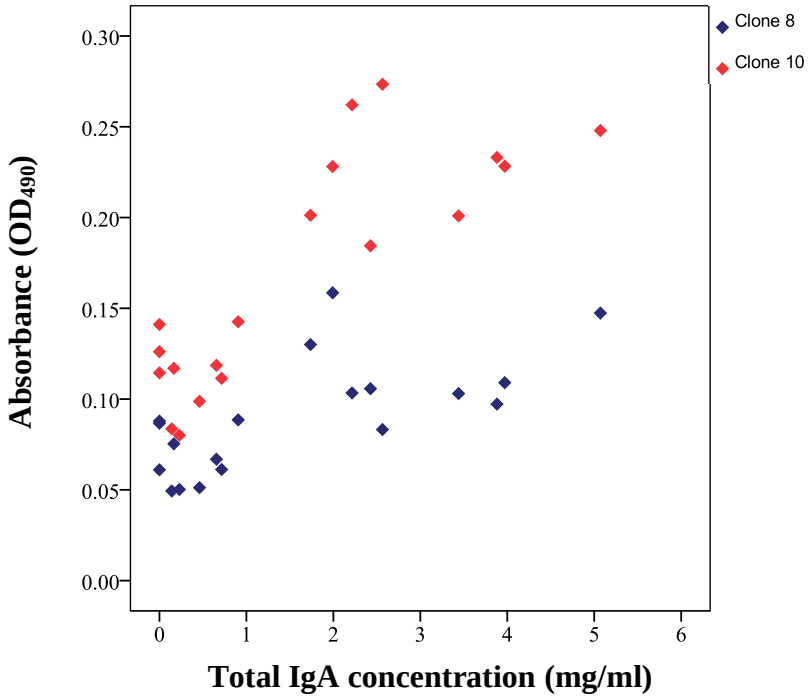

Figure 4.13 Relationship between levels of gastric lymph IgA binding to phage clones 8 and 10 selected by biopanning and the total gastric lymph IgA concentration at 7 dpc of sheep previously infected/challenged with *T. circumcincta* L_3.

Analysis of the association was conducted as above. Data points represent the absorbance values (OD) from individual gastric lymph samples (n=19). The absorbance values were plotted against the total IgA concentrations for the individual samples. The efferent gastric lymph was collected from sheep which had been previously infected with *T. circumcincta* L_3 and were subjected to a bolus challenge of 50,000 *T. circumcincta* L_3 ("GL-PI"), as in Fig. 4.13. Total IgA concentrations were published previously in Halliday *et al.* (2007).

Significant positive correlations (P=0.005 to P=0.01, R_s = 0.573 and 0.617) were found between the level of IgA binding to the peptide structures displayed by both phage clone 8 and 10 and the percentage of inhibited L_4 present in the abomasa and its contents at necrospy (Figure 4.14, Table 4.5).

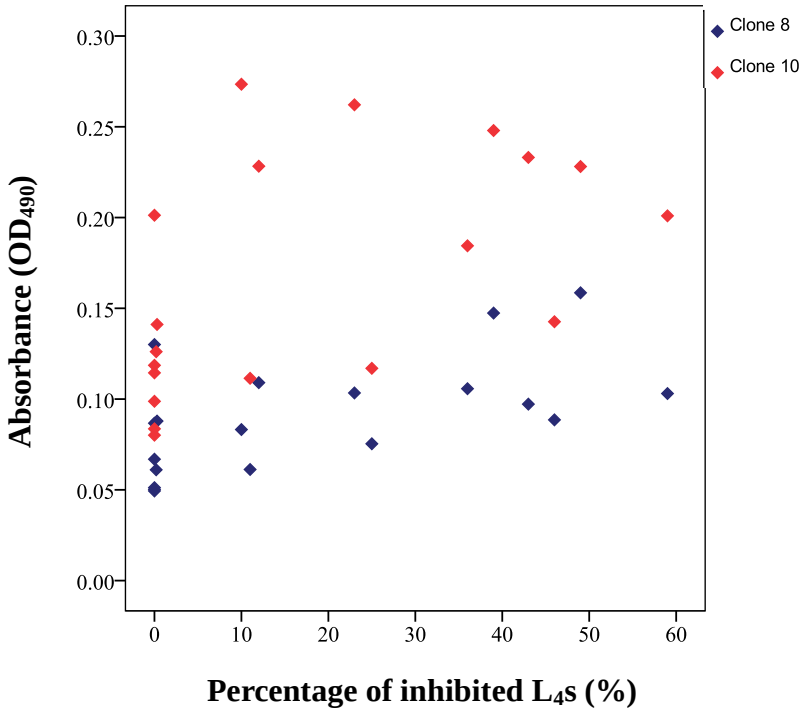

Figure 4.14 Relationship between the levels of gastric lymph IgA binding to phage clone 8 and 10 selected by biopanning and the percentage of inhibited *T. circumcincta* L_4 in the abomasa of sheep previously infected/challenged with *T. circumcincta*.

Analysis of the association was as in Fig 4.13. Data points represent the absorbance values (OD) in individual gastric lymph samples (n=19). The absorbance values were plotted against the percentage of inhibited L_4. Efferent gastric lymph was collected from individual sheep as detailed in Fig. 4.13. The percentages of inhibited L_4 present in the abomasum were estimated as detailed in Halliday *et al.* 2007.

4.4 Discussion

Random peptide phage display libraries are useful in the identification of sequences that can mimic epitopes; these 'mimotopes' do not necessarily bear primary sequence homology to native protein antigens but have potential in the development of novel peptide-based vaccines because they have potential to induce an immune response that mimics the immune response that would be induced by native antigens (Demangel *et al*., 1996; Narum *et al*., 2006). Here, we used purified IgA preparations to screen phage display libraries to target potential protective epitopes in somatic extracts of *T. circumcincta* L_3. A panel of phage clones was selected through high affinity for the IgA and analysis revealed a range of peptide sequences. These sequences had a number of characteristics which indicated that the peptides were potentially mimicking carbohydrate structures; aromatic ring structures, hydroxyl groups, hydrophobic residues. Work presented in the earlier Chapters revealed the importance of glycans on L_3 antigens in the immune response against *T. circumcincta*. Carbohydrates contain hydroxyl groups and carbon-sugar hydrophobic rings (for example, pentose which is a five-sugar ring and hexose which is a six-sugar ring), which allow the molecules to form hydrogen bonds with their ligands. For a peptide sequence to mimic the characteristics of carbohydrates it should contain residues with hydroxyl groups, hydrophobic residues and aromatic amino acids (Fukuda, 2006). The peptide sequences selected here contained a high proportion of aromatic amino acids and hydrophobic residues. Due to the molecular size and the hydrophobic properties of the aromatic ring this suggests that these peptides had the capability to form hydrogen bonds and therefore could emulate carbohydrate structures (Oldenburg *et al*., 1992; Scott *et al*., 1992). Previous studies indicated that aromatic amino acids appear with high frequency in peptide mimics of carbohydrates (Lang *et al*., 2006; Oldenburg *et al*., 1992). In a study which used phage display screening of a linear 7-mer library with a monoclonal antibody raised against the cross-reactive epitope (Gal-α1,3Gal) to identify peptide mimetics, aromatic amino acids occurred in selected peptides with a proportionally high frequency of 33% (phenylalanine, tyrosine and tryptophan) as well as residues with hydroxyl groups

(serine and threnonine: 36%) (Lang *et al*., 2006). In the human bacterial pathogen, *Shigella flexneri* (Phalipon *et al*., 1997), two monoclonal antibodies specific for the O-antigen in lipopolysaccharide of human serotype 5a were used to pan a 9-mer phage display library and 19 clones isolated were used in an immunisation and challenge trial in mice. In immunised mice, two of the clones induced anti-O-antigen-specific antibodies that were cross-reactive with *S. flexneri* native antigens (Phalipon *et al*., 1997). Taken together these results highlight the ability of phage display libraries to select peptide clones that mimic highly constrained structures such as carbohydrates. In vaccine development studies for the protozoan *Entamoeba histolytica*, monoclonal antibodies against surface proteophosphoglycans (PPGs), were used to pan six phage display libraries to reveal a single consensus motif (Gly-Thr-His-Pro-X-Leu) (Marinets *et al*., 1997; Melzer *et al*., 2002). Clones encoding this motif were able to induce a serum IgG response, specific to membrane antigens of *E. histolytica* trophozoites, following immunisation of the mice with whole phage particles (Melzer *et al*., 2002). The consensus peptide, GTHPXL, was synthesised as a peptide, conjugated to keyhole limpet haemocyanin (KLH) and used to immunise mice (Melzer *et al*., 2002). This construct induced high titres of anti-peptide antibodies and antibodies specific to *E. histolytica* PPGs and was protective against a challenge infection (Melzer *et al*., 2002).

Here, a panel of phage-displayed peptide sequences with a high level of binding to the affinity-purified IgA from *T. circumcincta* infected sheep was selected. Sequence analysis revealed that there were high proportions of aromatic and hydrophobic amino acid residues in these peptides, indicating that the peptide sequences might mimic carbohydrate structures on native antigens (Fukuda, 2006). The immunoreactivity of the peptide structures displayed by two of the phage clones, clones 8 and 10, was investigated by comparing peptide-specific IgA responses to known correlates of immunity to *T. circumcincta.* Mean clone 8 and 10 peptide-specific abomasal mucosal IgA levels were significantly higher ($P<0.01$) in the *T. circumcincta* trickle-infected/bolus-challenged sheep ("M-PI") compared to helminth-naive sheep (M-HF"). Positive associations for both clones were demonstrated between levels of peptide-

specific lymph IgA and the total IgA concentration in efferent gastric lymph at 7 dpc (P<0.01) and the percentage of inhibited L_4 present (P<0.01). Importantly, significant negative correlations between levels of phage clone-specific lymph IgA and total *T. circumcincta* burden were observed (P<0.05).

As polyclonal antibodies were used to screen the phage display library here, this was unlikely to result in the isolation of a single consensus sequence representing a conformational epitope (Prudencio *et al*., 2010; Tang *et al*., 2004). As expected, the peptide sequences obtained from biopanning (Table 4.2) did not contain a single consensus sequence or motif. These findings are similar to others in which polyclonal antibodies were used in biopanning experiments: for example in studies on the fluke, *F. hepatica* phage display panning of a 12-mer M13 filamentous library with polyclonal anti-*F.hepatica* native cathepsin L antibodies, a panel of seven clones was selected (Villa-Mancera *et al*., 2008). Immunisation with all phage particles in combination, without inclusion of adjuvant, induced higher IgG titres and reduced fluke burdens by 46-67%, compared to unvaccinated control sheep, after challenge with metacercariae (Villa-Mancera *et al*., 2008; 2010). Two selected clones (from the panel of seven used in these vaccination studies) were searched against the available peptide sequences for *F. hepatica* cathepsin L1 and L2 and revealed no matches in sequence, suggesting that the peptides recognised by the antibodies might mimic structural epitopes (Villa-Mancera *et al*., 2008; 2010).

Here, two TUPs (Ru *et al*., 2010; Vodnik, 2011) were identified. It is thought that such phage clones have accelerated growth and therefore dominate amplified phage. Two of the the TUP clones (2 and 6) were investigated for immunoreactivity: both bound IgA in abomasal mucus from previously infected/challenged sheep (Figure 4.8). Clone 6 was also bound by mucus IgA from sheep exposed to a single primary challenge infection of *T. circumcincta*. The analysis subsequently focussed on non-TUP clones (8 and 10) selected in the biopanning. In this study, a direct phage ELISA was developed. However there was a degree of inconsistency in the results obtained from this ELISA as there was a significant level of inter-plate variability (P=0.012). As a result, the direct

phage ELISA will require further optimisation to improve reliability and consistency between assay repetitions. The IgA-immunoaffinity-purified phage clones expressing heptameric peptides representing glycans on L_3 somatic antigens were used in ELISA experiments to assess the relationship between heptamer binding and correlates of immunity. Four parameters were analysed; total nematode burden, percentage of inhibited L_4, total IgA concentration in efferent gastric lymph and the lymphoblast response. Each of these has previously been correlated with development of protective immunity in *T. circumcincta*-infected sheep (Halliday *et al*., 2007). IgA in efferent gastric lymph bound the peptide structures in clones 8 and 10. IgA in these gastric lymph samples has been shown to bind L_4 somatic antigens (Halliday *et al*., 2007) and work presented here showed that the IgA also binds to L_3 antigens. The efferent gastric lymph samples from trickle-infected/bolus-challenged and primary bolus-infected sheep used in these experiments were from one single time-point, 7 dpc. This time-point corresponded to the peak in total IgA measured in these samples (Halliday *et al*., 2007). Here, significant positive correlations between total IgA concentration at 7 dpc and binding of heptamer-specific IgA to both phage clones 8 and 10 were evident (Figure 4.14). Previous studies into associations between the levels of *T.circumcincta* L3 antigen-specific abomasal mucus IgA and parameters of the immune response revealed a positive relationship between IgA binding and percentage of inhibited L_4 recovered from the abomasum (Beraldi *et al*., 2008; Stear *et al*., 1999). In the work here, levels of gastric lymph IgA specific to clones 8 and 10 were significantly positively correlated with percentage of inhibited L_4. A greater proportion of inhibited L4 in the abomasa of sheep infected with *T. circumcincta* will lead to a reduction in egg output and the level of pasture contamination. Therfore, a vaccination strategy directed to increasing the proportion of inhibited L_4 and reducing establishment of larvae in the abomasum will reduce the level of parasite exposure and the impact of parasitism on lambs turned out onto pasture.

In conclusion, two phage clones were selected that have the potential to mimic native epitopes on *T. circumcincta* L_3 antigens. The level of binding of efferent gastric

lymph IgA to the peptide structures displayed by both these phage clones was positively correlated to the percentage of inhibited L_4 present in the abomasum and negatively correlated to the total nematode burden. These associations indicate that the peptide structures displayed by the phage clones may imitate the structure of native epitopes on *T. circumcincta* L_3 antigens. A vaccination trial with helminth-naïve sheep will be the necessary next step to assess the level of protection afforded by the two phage clones. The two phage clones could be administered together or in separate formulations to sheep and to assess the level of protection three parasitological factors should be assessed; total nematode burden, faecal egg ouput and percentage of inhibited L_4, alongside the immunological parameters.

Chapter 5 : Structural epitope mimics of *T. circumcincta* L_3 native surface antigens

5.1 Introduction

In the development of a vaccine against parasitic nematodes, targeting incoming larvae is a sensible approach as this stage represents the parasites' first point of contact with the host. Studies suggest that antibodies in the local environment (i.e. abomasum) play an important role in naturally-induced immunity of sheep against *Teladorsagia circumcincta* (Balic *et al*., 2003; Bowles *et al.*, 1995). After ingestion from pasture, L_3 exsheath in the rumen of the sheep and then migrate to the abomasum within 3 dpi (Michel, 1974). The surface of *T. circumcincta* L_3 is therefore a major site for interactions between host defence mechanisms, such as local antibodies, and the incoming parasite (Ashman *et al*., 1995; Blaxter *et al*., 1992) and a potential focus for vaccine development as, theoretically, targeting this larval stage could reduce establishment of the parasite within the host (Meeusen, 1996; Newton and Meeusen, 2003).

Parasitic nematodes have an external surface structure, termed the cuticle, which is a complex multilayered structure with a unique composition (Blaxter *et al*., 1992). The epicuticle, the outermost layer, contains lipid components as well as proteins and glycoproteins, which are all accessible to antibody binding. The biophysical properties of the lipid-rich epicuticle may be altered during the transition from pre- to post-parasitic state following changes in the physico-chemical environment (Proudfoot *et al*., 1990; 1993). A carbohydrate-rich surface coat, termed the 'glycocalyx', is associated with the epicuticle and has been implicated in immune evasion (Maizels *et al*., 1993). Surface-associated antigens of numerous parasites have been shown to be stage-specific, including the bovine lungworm, *D. viviparus* and the abomasal parasite, *H. contortus* (Britton *et al*., 1993; Raleigh *et al*., 1996). Research into the cuticular proteins of *T. circumcincta* and *O. ostertagi* has revealed that, in all of the major developmental stages, surface polypeptides are expressed that are unique to each particular stage, suggesting that the cuticle is altered throughout the life cycle (Keith *et al*., 1990). This

suggests that the act of cuticle moulting during the parasitic stages is a useful tool to change the surface antigens exposed to the host environment and its immune system (Raleigh *et al*., 1996).

Surface antigens of *T. circumcincta* L_3, have been stripped from the parasites using detergent and subsequently used in an immunisation trial to investigate their ability to induce a protective response in sheep against challenge (Wedrychowicz *et al*., 1992; 1995). Sheep vaccinated with L_3 surface extracts in conjunction with a beryllium hydroxide adjuvant had a 72% reduction in worm burdens compared to the control sheep infected with a single bolus challenge of 50,000 L_3 (Wedrychowicz *et al*., 1992; 1995). Vaccination with L_3 surface extracts was also able to induce a humoral response, with vaccinated sheep having a higher level of L_3 surface antigen-specific serum IgA and IgG compared to control sheep (Wedrychowicz *et al*., 1992). In studies investigating the stage-specific expression of *H. contortus* antigens, antibody-secreting cell probes (ASCs) were generated from abomasal lymph nodes of sheep given an trickle infection with *H. contortus* L_3 followed by a challenge "bolus" dose of 50,000 L_3 (Bowles, Brandon and Meeusen, 1995). The ASCs were used to probe an immunoblot of an extract of L_3/L_4 *H. contortus* and the ASCs, derived from lymph nodes collected at 5 dpc, bound antigens present in the ranges 40-50 kDa and 70-83 kDa (Bowles, Brandon and Meeusen, 1995). Further localisation and purification experiments revealed that the ASC probes bound a glycoprotein-containing complex, termed *Hc-s*L3, from the surface of live *H. contortus* L_3 and that this complex could be shed from larvae following incubation with antibodies from the abomasum of sheep rendered immune by infection (Ashman *et al*., 1995; Bowles, Brandon and Meeusen, 1995). In an immunisation trial where sheep were immunised with *Hc-s*L_3, a significant response was measureable in immunised sheep following *H. contortus* L_3 challenge, with a 54% reduction in FWEC of immunised animals compared with control, adjuvant-only, sheep (Jacobs, Ashman and Meeusen, 1995). More recently, in a field trial sheep were immunised twice with *Hc-s*L_3 in conjunction with an aluminium hydroxide adjuvant, 4 weeks apart, and 4 weeks after the final immunisation were administered a challenge of 5000 *H. contortus*

L_3 (Piedrafita *et al*., 2012). Vaccination with the native *Hc*-sL_3 antigen showed reductions of 61% and 69% in FWEC and adult worm burdens, respectively, in vaccinated sheep compared to the adjuvant-only control sheep (Piedrafita *et al*., 2012). There was no evidence of a mucus antibody response to the vaccine antigens as no differences were detected in the levels of Hc-sL_3-specific antibodies, across all isotype groups, between immunised sheep and adjuvant-only control sheep (Piedrafita *et al*., 2012). In sheep which had been experimentally infected with a trickle infection and challenge regime with *Trichostrongylus colubriformis*, a specific, protective, mucus antibody response to the epicuticular glycan "CarLA" was evident (Harrison *et al*., 2003a; 2003b). CarLA homologues were subsequently found to be present on the exsheathed L_3 surface of a number of additional nematode species, including *T. circumcincta* and *H. contortus* (Maass *et al*., 2007). CarLA molecules have also been implicated in immune evasion (Maass *et al*., 2009). These previous studies highlight the importance of antigens displayed on the surface of infective larvae and the identification of native epitopes on these antigens could provide a viable and novel vaccine candidate target. Together, these studies highlight the potential of antigens present on the surface of parasitic nematode L_3 as viable targets for vaccine development.

As discussed in Chapter 4, phage display libraries are a useful tool in the identification of molecular interactions. Due to the difficulty in identification of conformational epitopes which play a role in the development of immunity (as discussed in Chapter 3), phage display libraries have been used to identify structures which mimic conformational epitopes (Coley *et al*., 2001; Gazarian *et al*., 2003). To date, a small number of studies have used phage display in the development of vaccines against parasitic helminths; for example, *Trichinella spiralis* (Gu *et al*., 2008) and *Fasciola hepatica* (Villa-Mancera *et al*., 2008). The study employing random peptide display libraries for *T. spiralis* vaccine development used a mouse monoclonal antibody, raised against a lead vaccine candidate, Ts87, isolated from adult *T. spiralis*, to bio-pan the library. It reported that immunisation of mice with two immunoreactive phage clones, followed by an oral challenge with 400 *T. spiralis* larvae 2 weeks after the final

immunisation, produced a significant reduction (29%) in worm burden compared to the unvaccinated animals (Gu *et al*., 2008). In the work detailed in Chapter 4, random peptide phage display libraries were used to identify phage-displayed peptide sequences which may mimic the structure of conformational epitopes found on antigens present in *T. circumcincta* L_3 somatic extract preparations. Two phage clones displayed promise as novel vaccine candidates because the level of peptide-specific IgA binding was associated with the three main known manifestations of immunity (Smith *et al*., 2009); total IgA concentration, inhibited larval levels and a reduction in worm burden.

The aims of the present Chapter were to:

i) Develop an immunofluorescence assay to ascertain if abomasal mucus antibody probes, derived from sheep which had been previously exposed to *T. circumcincta*, were able to bind directly to the surface of live, exsheathed *T. circumcincta* L_3.

ii) Employ phage display libraries to identify peptide sequences which mimic native structural epitopes displayed on the surface of exsheathed *T. circumcincta* L_3 by screening the libraries with mucus antibody preparations enriched for those antibodies which bound native antigens on the surface of live, intact exsheathed *T. circumcincta* L_3.

iii) Assess the relative immunoreactivity of selected peptide sequences from the phage clones by examining local antibody responses to them in sheep of varying levels of acquired immunity to *T. circumcincta* infection.

5.2 Materials and methods

5.2.1 Infection protocol for production of ovine abomasal mucus

All sheep used were Scotch Mules (Blackface ewe X Blue-faced Leicester ram), and were 6 months-old at the start of the trickle infection and 10 months-old at necroscopy. The sheep were raised indoors to reduce the risk of helminth exposure until the start of the experimental trickle infection. Lambs (n=4) were trickle-infected *per os* with 2,000 *T. circumcincta* L_3, three times per week for nine weeks (Figure 5.1).

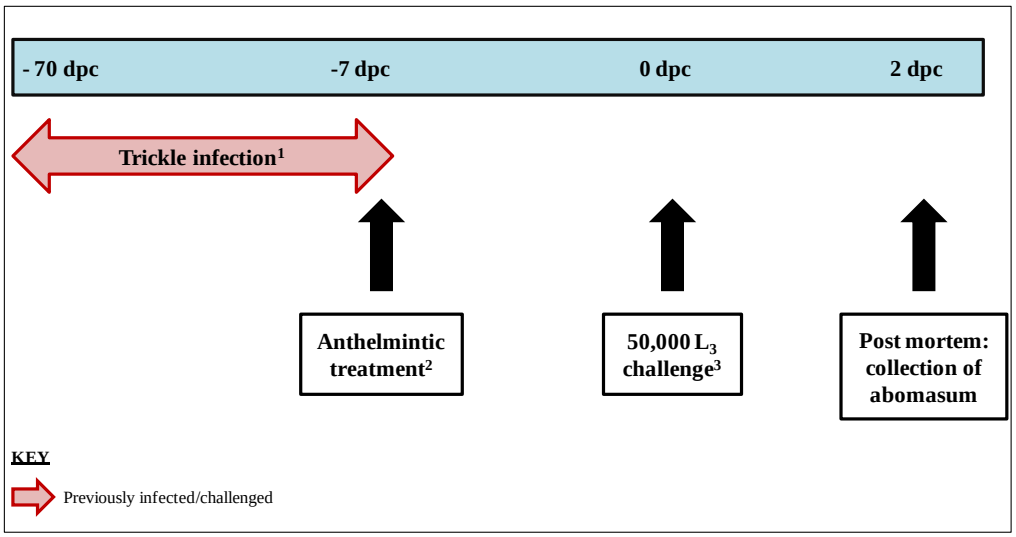

Figure 5.1 Design of experiment in sheep infected with *T. circumcincta* from which abomasal mucus was obtained for use as local antibody probes.

[1] Trickle infection: 2,000 *T. circumcincta* L_3 three times per week for nine weeks (red arrow).
[2] Fenbendazole (5mg/kg) administered seven days before challenge.
[3] Bolus of 50,000 *T. circumcincta* L_3 challenge administered on day 0.

Faecal worm egg counts (FWEC, see section 5.2.2) were performed on samples taken three times per week from the sheep, starting at 17 dpc until the end of the experiment. The anthelmintic fenbendazole (5mg/kg) was administered to remove any residual worms from the sheep before bolus challenge. Seven days later, the sheep were challenged *per os* with a single bolus dose of 50,000 *T. circumcincta* L_3. All animals

were killed by captive bolt and exsanguinated at 2 dpc, the abomasa were removed and processed for the collection of mucus as detailed in section 2.2.1.1.

5.2.2 Faecal worm egg counts

Faecal material was collected three times per week throughout the duration of the experiment. Upon collection, the faecal material was weighed and suspended in 10 ml of dH_2O per 10g of faeces. The faecal mixture was homogenized using a Stomacher ensuring that the faecal sample was thoroughly resuspended. A 10ml subsample was extracted, passed through a 1mm sieve into a pre-labelled beaker and the retentate washed through the sieve with 5 ml of dH_2O. The filtrate was transferred to a centrifuge tube and centrifuged at 112 *g* for 2 min to pellet the faecal material. The supernatant was removed and the faecal pellets resuspended in 10 ml of a saturated NaCl solution. The pellet was gently resuspended by inversion and the samples were centrifuged at 112 *g* for 2 min. Artery forceps were used to clamp the centrifuge tube below the meniscus and the upper volume transferred into a cuvette for egg counting. The upper chamber of the tube was rinsed with 1ml of saturated NaCl solution and this was also added to the cuvette. The cuvette was inverted several times, and a syringe used to add NaCl solution to create a positive meniscus before fixing the cuvette lid in place. Strongyloid eggs were counted with the aid of a graticule square across the transverse of the cuvette. The method for egg counting was as described in Christie and Jackson (1982) and is sensitive to 1 egg per gram (epg).

5.2.3 Detection of specific antibodies by ELISA

For a quantitative assessment of antigen-specific antibody responses in the previously infected sheep (section 5.2.1), the immunoreactivity of the abomasal mucus was investigated by ELISA. The method used was similar to that described in section 2.2.8, with some modifications. Briefly, microtitre plates were coated with 50µl of 5µg/ml *T.*

circumcincta extracts from L_3 and L_4 homogenates, in 50mM sodium bicarbonate, pH 9.6, and incubated at 4°C overnight. Plates were washed with PBST and blocked for non-specific binding as detailed in section 2.2.8. After re-washing, plates were incubated with 50µl of primary antibody (abomasal mucus from individual sheep, n=4), diluted at 1:5 in TNTT, and incubated for 2 h at room temperature. The secondary and tertiary antibody incubations were as detailed in section 2.2.8. All samples were measured in triplicate on each plate and each plate was repeated on two independent occasions. Negative controls omitting the primary antibody incubation step were included on each plate. A pool of efferent gastric lymph collected from sheep experimentally infected by a trickle infection/challenge protocol (detailed in section 2.2.1.2, termed GL-PI) was used as a positive control sample on all plates. This efferent gastric lymph was pooled from samples at specific time-points corresponding to the peak total IgA concentration, 6-10 dpc, as published in Halliday *et al*. (2007).

5.2.4 Western blotting

SDS-PAGE (section 2.2.6) was performed on *T. circumcincta* L_3 somatic extract (generated as described in section 2.2.4) and the separated proteins transferred by electroblotting onto a 0.45µM nitrocellulose membrane as described in section 2.2.7. The remaining steps in the blotting procedure were as in section 2.2.7 with the following modification: the primary antibody was sourced from abomasal mucus collected from each of the four sheep described in section 5.2.1.

5.2.5 Detection of ovine antibody binding to the surface of *T. circumcincta* L_3

Live *T. circumcincta* L_3 (*c.*10,000 larvae) were exsheathed by incubation in 750µl of 2% (w/v) sodium hypochlorite/16.5% sodium chloride for 3 min at room temperature. A sub-sample of larvae was examined under a microscope to ensure that 90% had exsheathed prior to the removal of the sodium hypochlorite. Exsheathed larvae were centrifuged at 112 *g* for 2 min. The larval pellet was washed with 10 ml dH_2O, centrifuged at 112 *g* for 2 min and the supernatant removed. This washing procedure

was repeated three times in total. Live exsheathed *T. circumcincta* L_3 (*c.* 2000) were transferred into a 1.5 ml tube. To block non-specific interactions, the larvae were incubated with 10% soya milk powder (Infasoy, Cow and Gate™) in PBS for 30 min at room temperature on a rotating roller mixer. Following blocking, larvae were washed in PBST by incubation of the samples on a rotating roller mixer for 2 min, centrifuged at 112 *g* for 3 min and the supernatant removed. The samples were washed three times. Larvae were incubated with 250 µl of primary antibody (a neat antibody extract preparation from abomasal mucus) from trickle-infected/challenged sheep (generated as described in section 5.2.1) or helminth-naïve sheep (generated as described in Section 2.2.1.1) for 90 min at 37°C on a rotating roller mixer. Larvae were re-washed three times with PBST and incubated with the appropriate secondary antibody according to the specific antibody isotype under investigation (Table 5.1) for 90 min at room temperature. The antibodies were diluted to the required concentration in PBST (Table 5.1). After the application of the final fluorescein isothiocyanate (FITC)-conjugated antibody (*i.e.* IgG, secondary; IgA, tertiary) larvae were rewashed in PBST (with 0.01% sodium azide). The larval pellet was resuspended in 50 µl of PBST and examined under a UV fluorescent microscope. Sheathed *T. circumcincta* L_3 were also processed in the same manner for comparison.

Isotype	**Secondary antibody**			**Tertiary antibody**		
	Description	**Manufacturer**	**Dilution**	**Description**	**Manufacturer**	**Dilution**
IgA	Mouse monoclonal anti-bovine/ovine IgA	Serotec, MCA628	1 in 250	Polyclonal rabbit anti-mouse immunoglobulins, FITC-conjugated	AbCam, AB8517	1 in 50
IgG	Monoclonal mouse anti-goat/sheep IgG, FITC-conjugated	Sigma, F4891	1 in 100	N/A	N/A	N/A

Table 5.1 Antibodies used for immunofluorescence assay investigating the immunoreactivity of *T. circumcincta* surface-exposed antigens against antibodies from abomasal mucus of sheep which had previously been infected with *T. circumcincta*.

5.2.6 Cross-reactivity of ovine abomasal mucus antibodies with other trichostrongylid nematodes

The cross-reactivity of ovine abomasal mucus (collected from sheep trickle-infected/challenged with *T. circumcincta*) against surface antigens from other nematode species was investigated using the immunofluorescence assay. *H. contortus* (*c*. 10,000) and *T. colubriformis* (*c*. 10,000) were exsheathed following the method outlined in section 5.2.5. Isolates of *H. contortus* and *T. colubriformis* larvae were kindly provided by Alison Morrison (Moredun Research Institute). Larvae were incubated with the antibodies for detection of both IgA and IgG binding as detailed in section 5.2.5.

5.2.7 Elution of L_3 surface-bound antibodies

Following the incubation of *T. circumcincta* L_3 (*c*. 50,000) with 5 ml of primary antibody from trickle-infected/challenged sheep (sections 5.2.1 and 5.2.5) bound antibodies were eluted from the L_3 surface. Larvae were washed with PBST, centrifuged at 112 *g* for 3 min, rewashed in PBST and centrifuged again, then, following removal of the PBST, incubated with 500 µl of 0.1M glycine-HCl, 6M urea, pH 2.5 for 5 min on a rotating rotary mixer. The samples were centrifuged at 400 *g* for 2 min, the supernatant containing eluted antibodies transferred to a fresh 1.5 ml tube and the pH of the elution buffer immediately readjusted to pH7 with 75 µl of 1M Tris, pH 9.1. Sub-samples of larvae were taken pre- and post-elution and processed accordingly for the immunofluorescence assay to detect IgA and IgG binding to the surface antigens and to illustrate elution of the surface-bound antibodies.

5.2.8 Confirmation of reactivity of antibodies purified directly against the surface of exsheathed *T. circumcincta* L_3

Binding of L_3-surface purified antibodies (section 5.2.5 and 5.2.7) to antigens present in L_3 extracts was investigated by immunoblotting. Briefly, 5 µg/lane of *T. circumcincta* L_3 S1 and S2 extracts (section 2.2.3) were run on 4-12% NuPAGE SDS gels under reducing conditions (section 2.2.4). Once run, one lane of the gel was stained using

Simply Blue Stain and the remaining lanes electro-blotted onto a 0.45 µm nitrocellulose membrane (section 2.2.5). To block non-specific binding sites, nitrocellulose membranes were incubated in TNTT overnight at 4°C. The appropriate lanes were then incubated with antibodies purified against the surface of exsheathed *T. circumcincta* L_3 (see section 5.2.7 and Table 5.2) for 2 h at room temperature. Lanes were then washed in TNTT for 10 min, repeated 3 times. Secondary and tertiary antibodies were then applied (Table 5.2) for 1 h at room temperature, prior to 3 x 10 min TNTT washes. Following the final wash after incubation with the HRP-conjugated antibody, detection was carried out using DAB (Sigma).

Primary		**Secondary**			**Tertiary**		
	Dilution	**Description**	**Manufacturer**	**Dilution**	**Description**	**Manufacturer**	**Dilution**
IgA[1]	1 in 10	Mouse monoclonal anti-bovine/ovine IgA	Serotec, MCA628	1 in 250	Polyclonal rabbit anti-mouse Igs, HRP-conjugated	Dako, P0260	1 in 1000
IgG[1]	1 in 10	Monoclonal mouse anti-goat/sheep IgG, HRP-conjugated	Sigma, A0452	1 in 1000	N/A	N/A	N/A

Table 5.2 Antibodies used for the detection of ovine IgA and IgG on immunoblots.

[1]IgA and IgG in abomasal mucus of sheep with an experimental infection of *Teladorsagia circumcincta*. The antibodies in this mucus were purified directly against the surface of live exsheathed *T. circumcincta* L_3.

Immunofluorescence was used to confirm that the reactivity of antibodies purified from the surface of exsheathed *T. circumcincta* L_3 was retained following the elution steps. Live, exsheathed *T. circumcincta* L_3 (section 5.2.5) were processed for the larval immunofluorescence assay as detailed in section 5.2.5 with the following modification. Following the blocking steps and washing, larvae were incubated with 250 µl of antibodies purified directly against the surface of exsheathed *T. circumcincta* L_3 (generated as described in sections 5.2.5 and 5.2.7) for 90 min at 37°C on a rolling rotator mixer. Following the primary antibody incubation step, the samples were incubated with the developing antibodies as outlined in section 5.2.5 and Table 5.2.

5.2.9 Phage display library panning

Abomasal mucus antibodies purified against surface antigens on *T. circumcincta* L_3 were used for screening commercially available heptapeptide (PhD-7™) M13 pIII libraries (New England Biolabs Inc, USA). L_3-surface purified antibodies (10µg/ml), prepared as described in section 5.2.7, were diluted in coating buffer (0.1M sodium carbonate, pH 8.6) and used to coat a 90mm Petri dish. Phage binding was then performed according to the manufacturer's instructions and as detailed in section 4.2.2. The eluted phage were amplified, as described in section 4.2.2.1, prior to use as the input phage for the subsequent rounds of panning with three complete rounds of panning conducted in total. For calculation of the plaque-forming units for both the unamplified panning eluates and amplified phage culture supernatants, the phage were titrated in ER2738 as described in section 4.2.2.1. Selected, antibody-binding, phage clones were used for DNA sequencing and for target specificity confirmation by phage ELISA. Fifty clones were selected and isolated for initial DNA sequencing. Subsequently, 20 of these sequenced clones also had a phage stock prepared for assessment of target specificity by ELISA. Individual phage clones were amplified from the phage stock in accordance to the protocol described in section 4.2.2.1 to generate material for the DNA preparation and phage ELISA. Phage DNA sequencing templates for the fifty clones were prepared following the methods outlined in section 4.2.2.3. The DNA sequences obtained from the sequence analysis of the immunoreactive heptapeptides were analysed as described in section 4.2.2.4. Bioinformatic analysis of the resulting peptide sequences was also conducted as described in section 4.2.2.5.

5.2.10 Assessment of specificity of phage clones for target antibodies by ELISA

The target specificities of selected phage clones were assessed by distinguishing true target binding from background binding to both the plastic support and the BSA blocking agent. This was achieved through comparison of the level of phage binding in uncoated wells of the 96-well plate to wells coated with the antibodies purified against L_3 surface-exposed antigens (as illustrated in Figure 4.4). The amplified phage stocks

of 20 clones (see section 5.2.9) were re-amplified to obtain sufficient quantities for the assays. Amplification of the individual phage stocks was achieved by following the method outlined in section 4.2.5. The target specificities of the 20 individual phage clones were investigated by following the phage ELISA method detailed in section 4.2.5. Briefly, for each phage clone, three wells of the 96-well plate were coated with 100 µl of L_3-surface purified antibodies (purified by the method described in sections 5.2.5 and 5.2.7) at 100µg/ml in 0.1M sodium carbonate, pH 8.6. Three wells of the 96-well plate were left uncoated for each clone and the plates were incubated overnight at 4°C. The following steps for washing, phage incubation and the detection of antibody-phage binding were as detailed in section 4.2.5. For each phage clone, the OD values obtained from the wells coated and uncoated with the target were compared.

5.2.11 Phage ELISAs probed with efferent gastric lymph IgA from sheep with differing levels of immunity to *T. circumcincta*

The ovine antibody probes from efferent gastric lymph used here were described in section 2.2.1.2 and had been collected from sheep which had received a trickle infection and bolus challenge of *T. circumcincta* L_3 (see section 2.2.1.2). The codes used for these samples are detailed in Table 5.3.

Sample	Infection status	Abbreviation
Efferent gastric lymph	Previous trickle infection/challenge	GL-PI
	Challenge only	GL-CO
	Helminth-naive	GL-N

Table 5.3 Summary and abbreviations for samples used in immunoreactivity investigations.

Key: GL = efferent gastric lymph; PI = previously infected by a trickle infection/challenge; CO = primary infection with a single bolus challenge; N = helminth-free naive sheep.

Four phage clones (Clone 1, WTPSVRP; Clone 3, WPTLQWA; Clone 12, SWPQRTN; Clone 16, GWPKFTK) were investigated to quantify binding of IgA in efferent gastric lymph from previously infected/challenged sheep to the structures presented by the peptide sequences. Further analysis was conducted to identify if binding to the peptide clones was related to known correlates of immunity: worm burden, percentage of inhibited *T. circumcincta* L_3 and total concentration of IgA in efferent gastric lymph at 7

dpc. Following the optimisation procedure described in section 4.2.7, the "Direct phage ELISA" was employed. Briefly, microtitre plates were coated with an individual phage clone diluted to 10^{12} pfu/ml in coating buffer (50µl/well) overnight at 4°C. Following washes in PBST, primary antibody incubations were conducted at room temperature for 1 h. The sources of primary antibodies were individual efferent gastric lymph samples collected at a single-time point during experimental infection: "G-PI" at 7 dpc, "GL-CO" at 7 dpc and "GL-HF" at 0 dpc (Table 5.3). All remaining procedures were as documented in the optimised direct phage ELISA described in section 4.2.7 (b). All primary antibody sample/phage clone combinations were conducted in duplicate on each plate. Each plate was repeated on three independent occasions. The absorbance values presented were from data generated across the six readings and are presented as mean with the standard deviation.

5.2.12 Statistical analysis of immunorecognition ELISAs – correlations

The data obtained from both the assays investigating the level of ovine IgA binding to the four phage clones and the relationships between the level of IgA binding and known immune parameters were not normally distributed (Anderson Darling Test, $p<0.05$). The mean values obtained in the ELISAs investigating the level of ovine IgA binding to the four phage clones/peptide structures from the different infection groups were statistically evaluated by non-parametric ANOVA and a GLM. Relationships between the levels of IgA binding to the selected peptide sequences and known immune parameters were summarised by non-parametric correlation analysis using Spearman rank correlation coefficient (SPSS version 19). For all statistical tests, the level of significance was set at $p<0.05$.

5.3 Results

5.3.1 Parasitological and immune responses against larval antigens in abomasal mucus from donor sheep

During the trickle infection programme the FWEC steadily decreased (Figure 5.2). At day 61, an egg count of less than 50 eggs per gram (epg) was obtained in all animals. FWECs were 0 epg following the anthelmintic treatment (Figure 5.2).

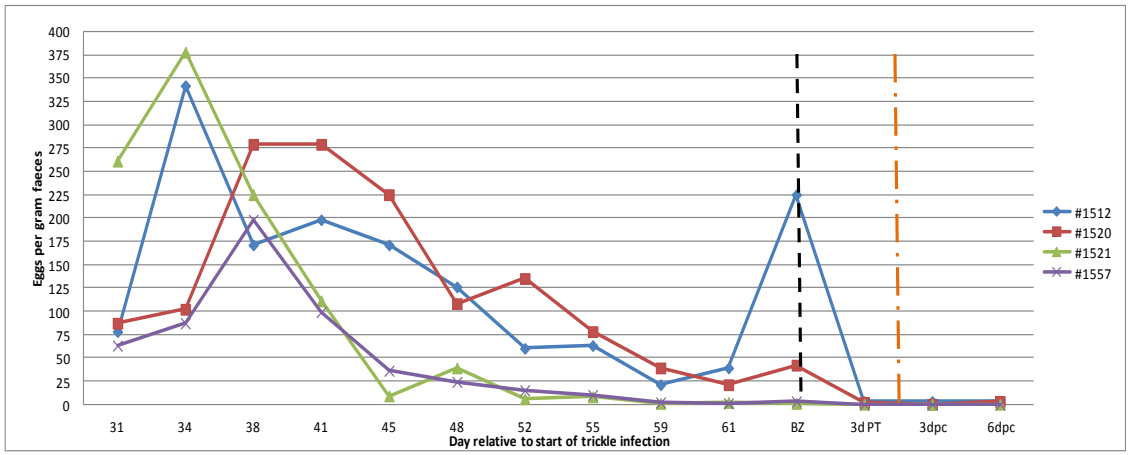

Figure 5.2 Faecal egg counts in lambs experimentally infected with *T. circumcincta* L_3 through an experimental trickle infection and in response to a single 50,000 L_3 bolus challenge.

FWEC were performed in duplicate for each animal and the mean value obtained as the eggs per gram of faeces is plotted for each individual animal. The vertical black dashed line represents the point ("BZ" on the x axis) in the infection programme at which the trickle infection ceased and all of the animals (n=4) were administered the anthelmintic, fenbendazole (5mg/kg), to remove residual *T. circumcincta*. The vertical orange dashed line represents the point at seven days post-treatment when all sheep were given a single bolus challenge of 50,000 *T. circumcincta* L_3. FWEC were then monitored for a further 6 days before the animals were euthanased to retrieve the abomasal mucus.

ELISAs were used to investigate abomasal mucus antibody isotypes that bound the *T. circumcincta* L_3 antigens. Abomasal mucus IgA and IgG activity against L_3 somatic antigens was detected in the samples collected from the four previously infected/challenged sheep (Figure 5.3), with some degree of individual variation between animals.

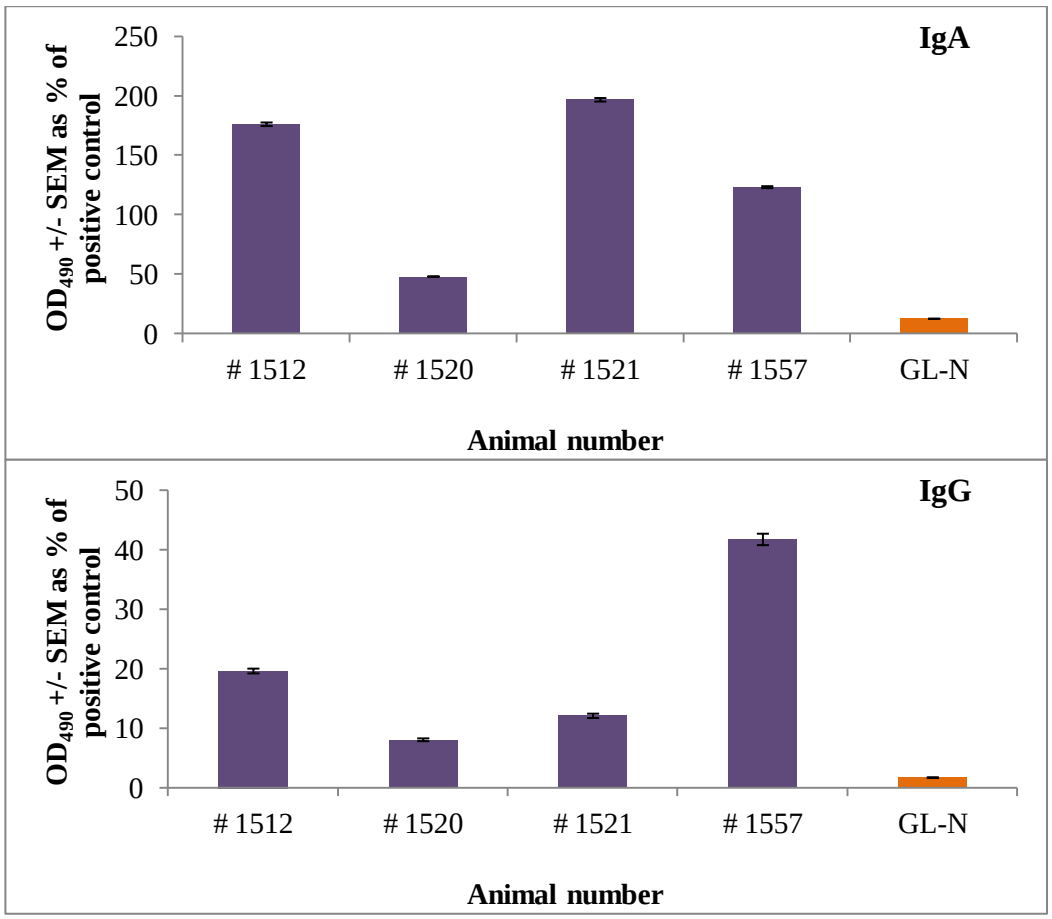

Figure 5.3 *Teladorsagia circumcincta* L_3 somatic antigen-specific abomasal mucus IgA and IgG levels in sheep previously infected/challenged with the parasite in an experimental infection regime.

The abomasal mucus samples were obtained from individual sheep which had received a trickle infection and single bolus challenge infection. Group "GL-N" represents pooled efferent gastric lymph from helminth-naïve sheep and was used as a 'negative control'. The positive control was pooled efferent gastric lymph collected over 6-10 dpc from previously infected/challenged sheep. The mean OD (± standard error of mean (SEM), n=6, results are expressed as a percentage of the positive control.

5.3.2 Detection of ovine antibody binding to the surface of *T. circumcincta* L_3

Following incubation of live, ensheathed *T. circumcincta* L_3 in the primary antibody source (i.e. abomasal mucus or PBS) for 90 min at 37°C, the majority of the larvae in the sample had exsheathed (a representative examples is shown in Figure 5.4). This was

observed in all the primary incubations conditions using (a) abomasal mucus from previously infected/challenged sheep, (b) abomasal mucus from helminth-naïve sheep and (c) no primary antibody control (PBS only). As a result all subsequent assays to determine binding of ovine mucus antibodies to *T. circumcincta* larvae were conducted with *T. circumcincta* L_3 which had previously been exsheathed with sodium hypochlorite (Section 5.2.5).

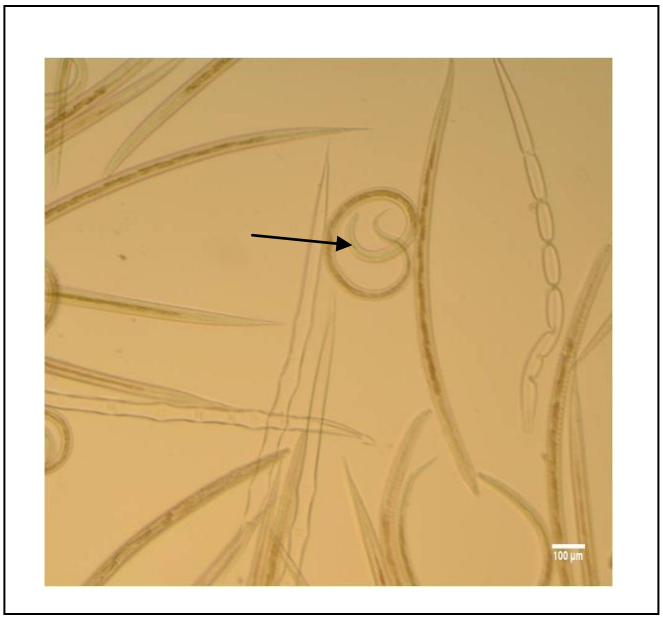

Figure 5.4 Image of *T. circumcincta* L_3 which had exsheathed during incubation with primary antibody source in PBS

Live, sheathed *T. circumcincta* L_3 were incubated at 37°C for 90 min in a 1.5ml Eppendorf tube with PBS. Following incubation, the larvae were examined under a microscope. A representative example of larvae is shown and the arrow depicts the discarded sheath of *T. circumcincta* L_3.

IgA in abomasal mucus collected from sheep previously infected/challenged with *T. circumcincta* L_3 bound antigens present on the surface of live, exsheathed *T. circumcincta* L_3 (Figure 5.5, panel B). No IgA binding was observed in mucus from helminth-naïve sheep or in the no primary antibody control (Figure 5.5, panels A and C, respectively). *T. circumcincta* infective larvae showed a degree of internal autofluorescence at the emission and excitation wavelengths used to detect the FITC conjugated antibody; this autofluorescence was seen in the larvae incubated with PBS only (Figure 5.5, panel C).

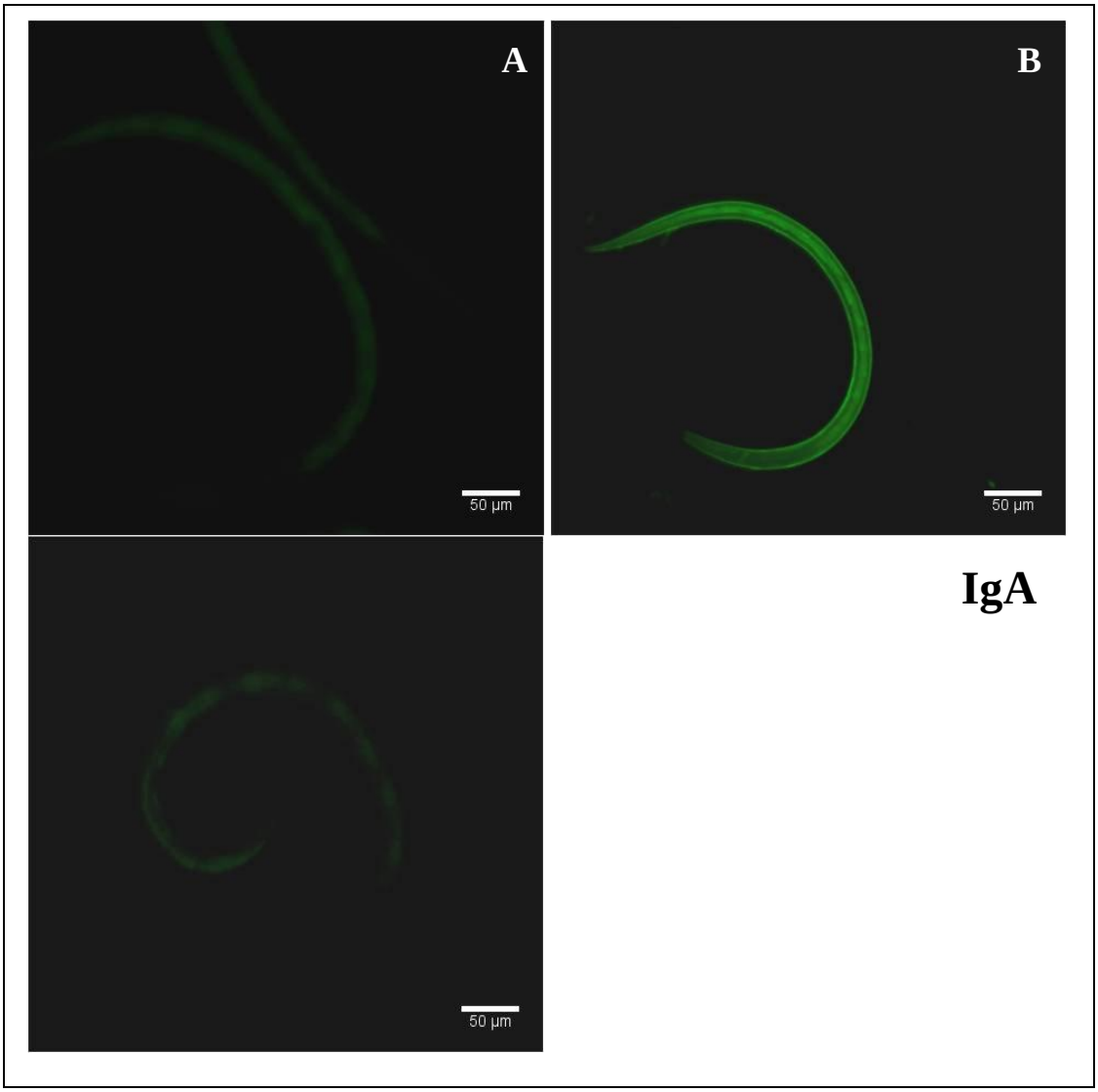

Figure 5.5 Immunofluorescent staining of *T. circumcincta* L_3 larvae with IgA from abomasal mucus obtained from both trickle-infected/challenged and helminth-naïve sheep

Ovine IgA binding to *T. circumcincta* L_3 surface antigens was detected with a combination of monoclonal mouse anti-bovine/ovine IgA and polyclonal anti-mouse immunoglobulins-FITC conjugated. Exsheathed *T. circumcincta* L_3 were incubated with mucus from (a) helminth-naïve sheep, (b) trickle-infected/challenged sheep and (c) PBS only (no primary control).

IgG in abomasal mucus obtained from sheep previously infected/challenged with *T. circumcincta* L_3 bound the surface of exsheathed L_3 (Figure 5.6, panel B). *T. circumcincta* L_3 incubated with abomasal mucus from helminth-naïve sheep or PBS did not bind IgG (Figure 5.6, panels A and C, respectively).

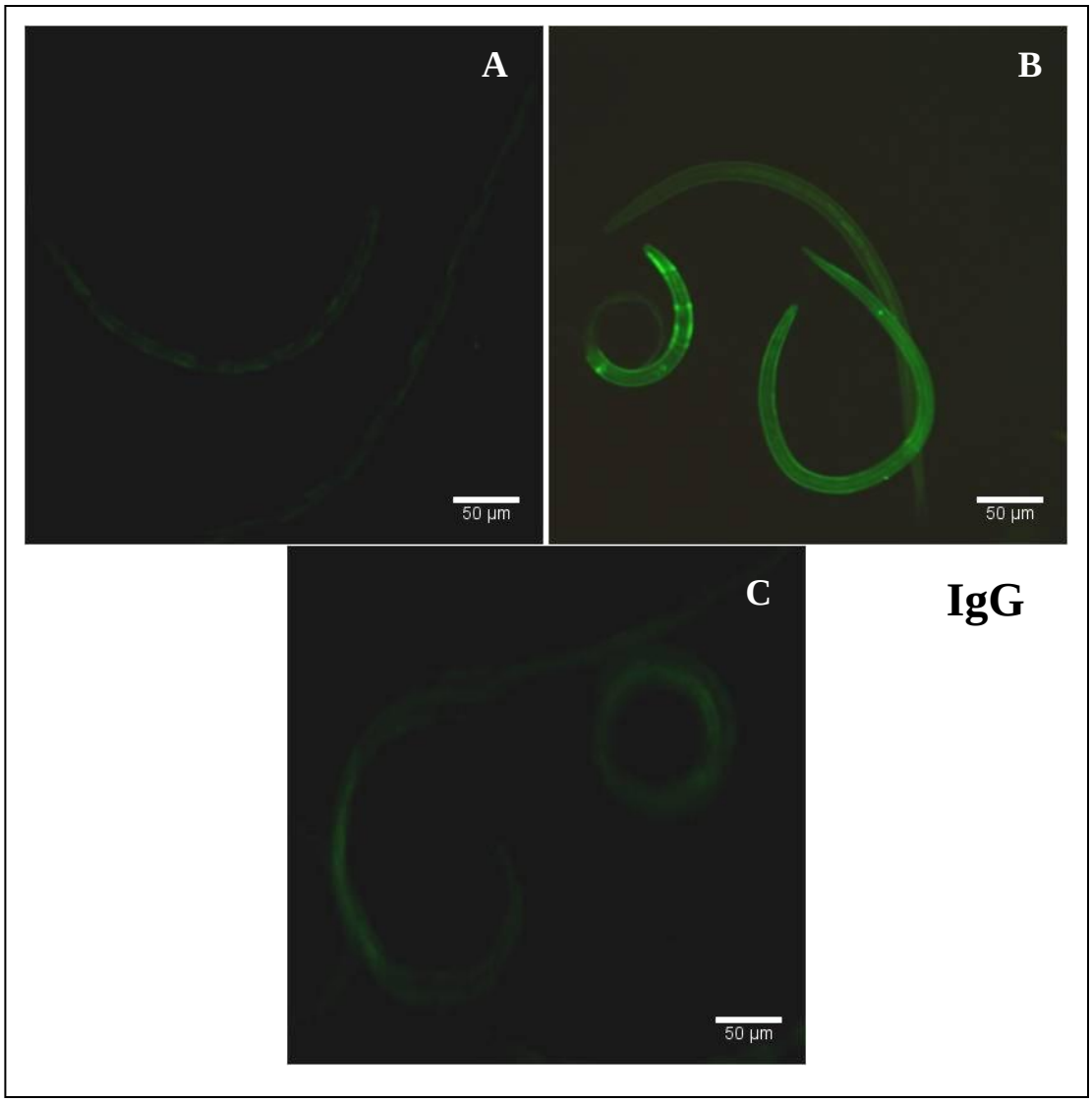

Figure 5.6 Immunofluorescent staining of *T. circumcincta* L_3 larvae with IgG from abomasal mucus obtained from both trickle-infected/challenged and helminth-naïve sheep.

Ovine IgG binding to *T. circumcincta* L_3 surface antigens was detected with a monoclonal mouse anti-goat/sheep IgG-FITC antibody. Exsheathed *T. circumcincta* L_3 were reacted with mucus from (a) helminth-naïve sheep, (b) trickle-infected/challenged sheep and (c) PBS only (no primary control). *T. circumcincta* L_3 were exsheathed *in vitro* through exposure to sodium hypochlorite solution and were exsheathed prior to primary antibody incubations.

5.3.3 Cross reactivity of ovine abomasal mucus antibodies with other trichostrongylid nematodes

The immunofluorescence assay was adapted to investigate cross-reactivity of abomasal mucus IgA and IgG obtained from sheep previously infected with *T. circumcincta* L_3 with antigens on the surface of the L_3 stages of other trichostrongylid nematode species. IgA in the abomasal mucus of sheep experimentally trickle-infected/bolus-challenged with *T. circumcincta* L_3 displayed cross-reactivity to antigens present on the surface of exsheathed *H. contortus* (Figure 5.7, panel A) and *T. colubriformis* L_3 (Figure 5.7, panel B). There was no reactivity of abomasal IgA obtained from helminth-naïve sheep against L_3 surface antigens of *H. contortus* (Figure 5.7, panel C) or *T. colubriformis* (Figure 5.7, panel D). Again, exsheathed *H. contortus* and *T. colubriformis* L_3 larvae expressed a degree of internal fluorescence when larvae were incubated with the no primary antibody control (Figure 5.7, panels E and F).

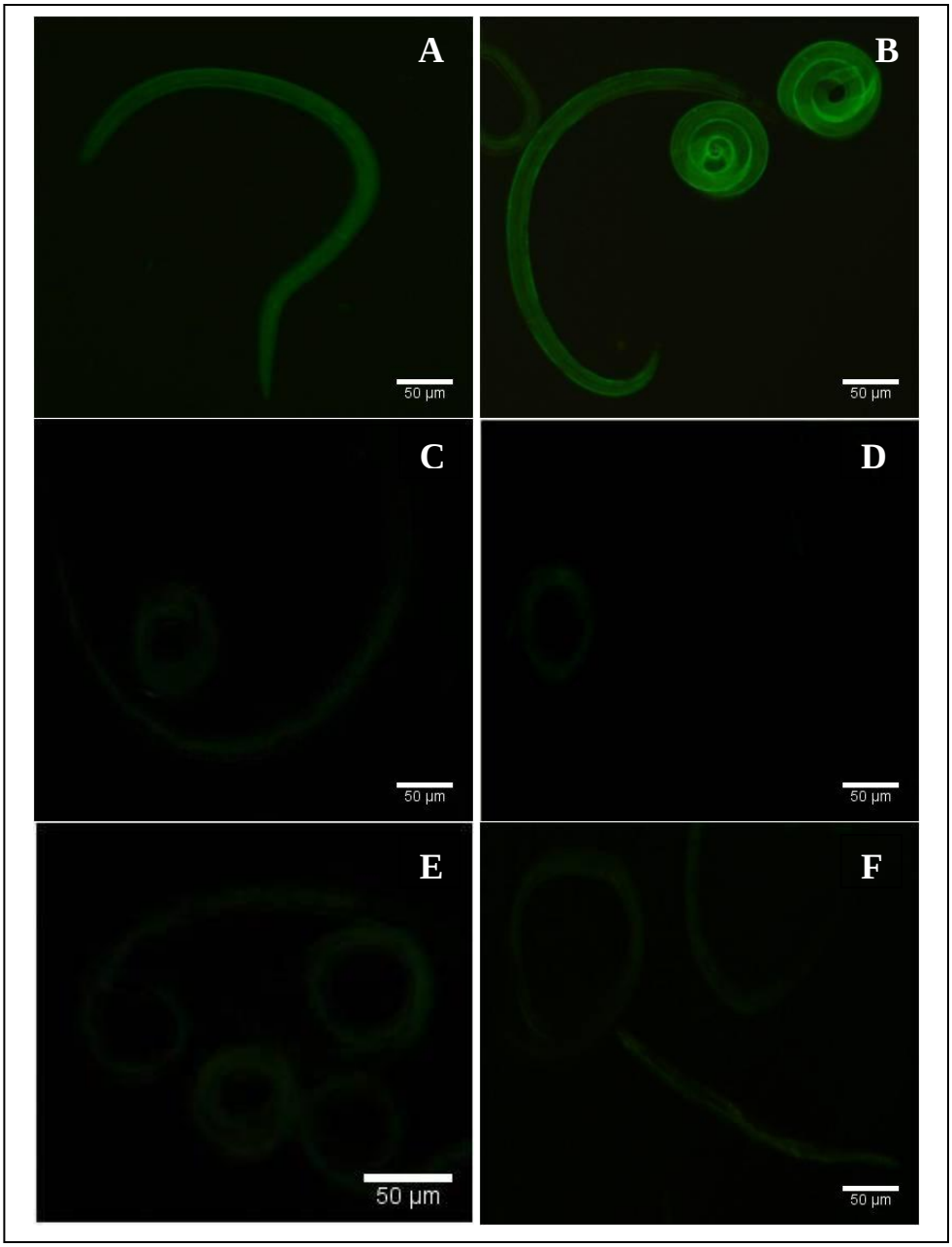

Figure 5.7 Immunofluorescent staining of exsheathed *H. contortus* and *T. colubriformis* L_3 with IgA from abomasal mucus collected from sheep which had been subjected to a trickle infection/challenge with *T. circumcincta* L_3.

Ovine IgA binding to trichostrongylid nematode surface antigens was detected with a combination of monoclonal mouse anti-bovine/ovine IgA and polyclonal anti-mouse immunoglobulins-FITC conjugated. Exsheathed *H. contortus* (A) and *T. colubriformis* (B) L_3 were incubated with abomasal mucus from sheep subjected to an experimental trickle infection and a single bolus challenge of 50,000 *T. circumcincta* L_3. Exsheathed *H. contortus* (C) and *T. colubriformis* (D) L_3 were incubated with abomasal mucus from helminth-naïve sheep. No primary antibody control: Samples of *H. contortus* (E) and *T. colubriformis* (F) were incubated with PBS only.

Abomasal IgG in sheep given an experimental trickle infection/challenge with *T. circumcincta* L_3, also bound to antigens present on the surface of exsheathed *H. contortus* and *T. colubriformis* L_3 (Figure 5.8, panels A and B). No IgG binding was detected when *H. contortus* and *T. colubriformis* L_3 were incubated with abomasal mucus from helminth-naïve sheep or PBS alone (Figure 5.8, panels C–F).

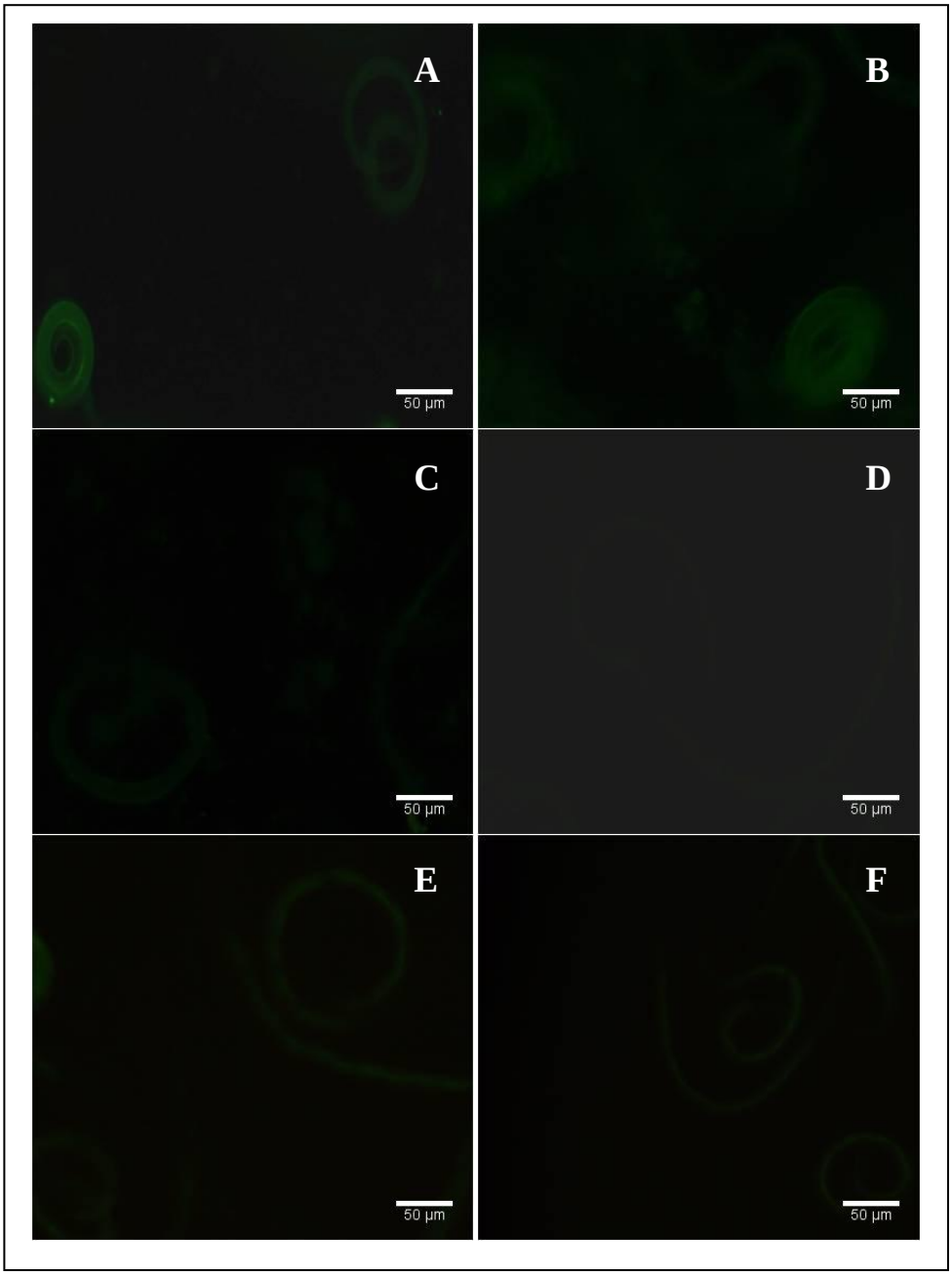

Figure 5.8 Immunofluorescent staining of exsheathed *H. contortus* and *T. colubriformis* L_3 with IgG in abomasal mucus from sheep subjected to a trickle infection/challenge with *T. circumcincta* L_3.

IgG binding to nematode surface antigens was detected with a combination of monoclonal mouse α-bovine/ovine IgA and polyclonal anti-mouse immunoglobulins-FITC conjugated. Exsheathed *H. contortus* (A) and *T. colubriformis* (B) L_3 were incubated with abomasal mucus from sheep subjected to an experimental trickle infection and a single bolus challenge of 50,000 *T. circumcincta* L_3. Exsheathed *H. contortus* (C) and *T. colubriformis* (D) L_3 were incubated with abomasal mucus from helminth-naïve sheep. No primary antibody control: samples of *H. contortus* L_3 (E) and *T. colubriformis* (F) were incubated with PBS only.

5.3.4 Elution of bound antibody from surface of exsheathed *T. circumcincta* L_3

To determine if the procedure used to elute antibody bound to the surface of exsheathed *T. circumcincta* L_3 was successful, immunofluorescent labeling of antibodies on the larval surface before and after elution was performed. In Figure 5.9, panel A shows *T. circumcincta* L_3 incubated with abomasal mucus from previously infected/challenged sheep (generated as detailed in Section 5.2.1) and subsequently probed with a FITC-conjugated antibody to detect IgA binding to the L_3 surface antigens. In panel B, the larvae were incubated with the same primary antibody source used in panel A (i.e. abomasal mucus from previously infected/challenged sheep) and, following this incubation, bound antibodies were eluted as detailed in section 5.2.7, before incubation of the larvae with anti-ovine IgA or IgG FITC-conjugates. The length of exposure for the capture of the immunofluorescence images was set to 13 secs for all samples to allow direct comparison of intensity of fluorescence between samples. Intensity of fluorescence on larvae probed for IgA binding post-elution was clearly reduced when compared with pre-elution L_3 samples, demonstrating successful elution; however, there was no obvious difference in immunofluorescence between pre- and post-elution L_3 when examined for IgG binding (Figure 5.9, panels C and D).

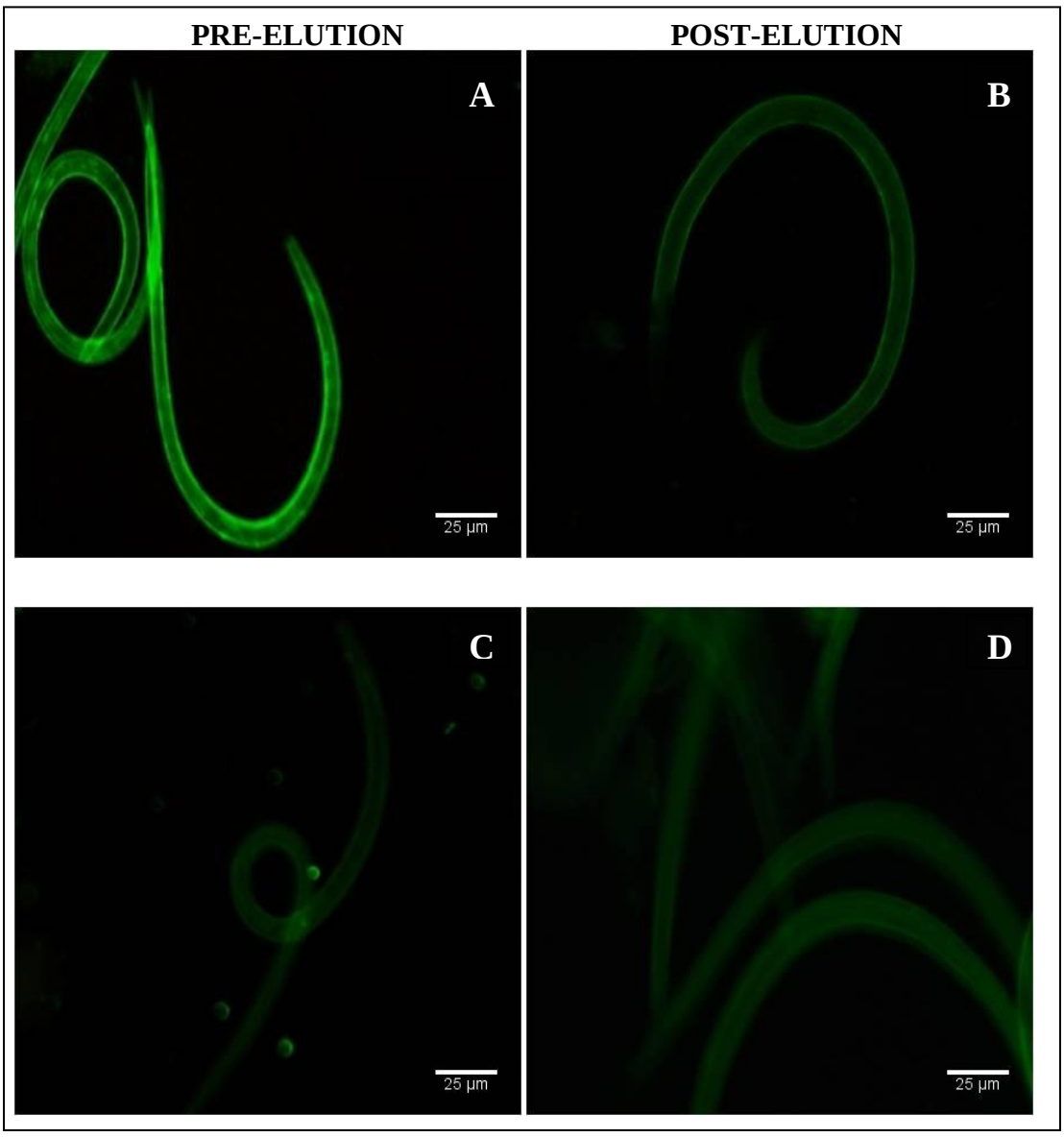

Figure 5.9 Comparison of immunofluorescent staining of *T. circumcincta* L_3 with abomasal mucus IgA and IgG obtained from sheep infected with *T. circumcincta*, pre- and post-elution of antibodies bound to surface antigens.

Exsheathed *T. circumcincta* L_3 were incubated with abomasal mucus from sheep subjected to an experimental trickle infection and single bolus L_3 challenge and probed for IgA binding (A) and IgG binding (C). Antibodies bound to the surface antigens of L_3 were eluted with 0.1M glycine/6M urea buffer and then re-probed for IgA (B) and IgG binding (D). Ovine IgA binding to *T. circumcincta* L_3 surface antigens was detected with a combination of monoclonal mouse anti-bovine/ovine IgA and polyclonal α-mouse immunoglobulins-FITC-conjugated antibodies. Ovine IgG binding was detected with a monoclonal mouse anti-goat/sheep IgG-FITC-conjugated antibody.

Following elution of antibodies, it was necessary to investigate if these were still able to bind to the surface of fresh exsheathed *T. circumcincta* L_3, so a further round of incubation and immunofluorescent detection of antibody binding was undertaken using the surface-purified, eluted antibodies as the primary antibody source. Surface-purified, eluted IgA was still able to bind *T. circumcincta* L_3 surface antigens (Figure 5.10, panel A) and surface-purified, eluted IgG binding to *T. circumcincta* L_3 was detected on some larvae (Figure 5.10, panel C).

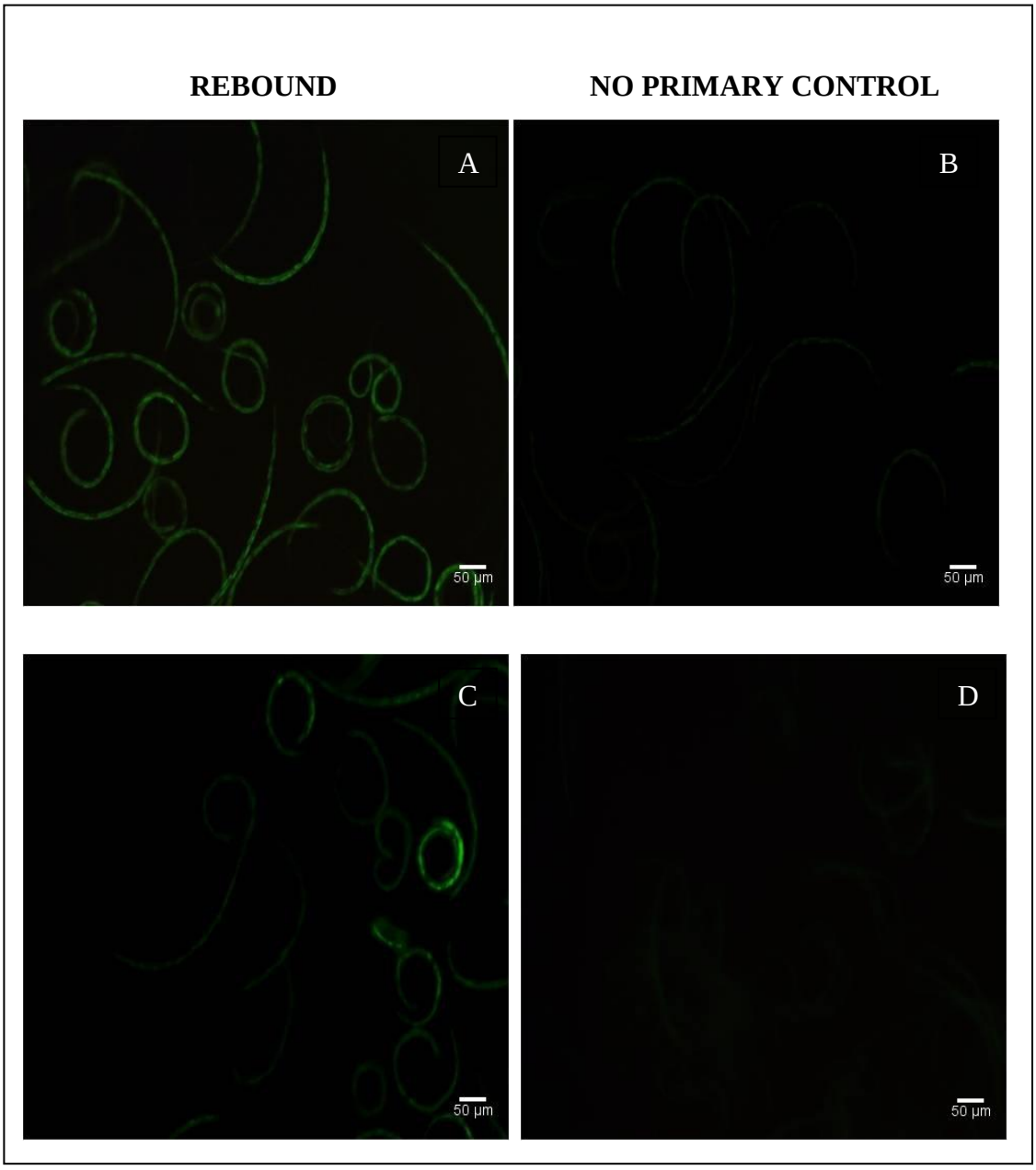

Figure 5.10 Re-binding of *T. circumcincta* L_3 surface-affinity purified abomasal mucus antibodies purified to exsheathed L_3.

Approximately 1,000 exsheathed *T. circumcincta* L_3 were incubated with abomasal mucus antibodies purified against the surface of exsheathed *T. circumcincta* L_3 and probed for IgA binding (panel A) and IgG binding (panel C). No primary antibody controls contained approximately 1, 000 *T. circumcincta* L_3 incubated with PBS and probed for IgA (panel B) and IgG binding (panel D). Ovine IgA binding to *T. circumcincta* L_3 surface antigens was detected with a combination of monoclonal mouse anti-bovine/ovine IgA and polyclonal anti-mouse immunoglobulins-FITC conjugates. Ovine IgG binding to *T. circumcincta* L_3 surface antigens was detected with a monoclonal mouse anti-goat/sheep IgG-FITC antibody.

5.3.5 Binding of surface-purified, eluted antibody to *T. circumcincta* L_3 antigens

Immunoblotting demonstrated that IgA in the surface-purified, eluted antibodies (see Sections 5.3.4) bound antigens in *T. circumcincta* S1 and S2 somatic extracts (Figure 5.11, Lanes 1 and 5, respectively). In the lane of S1 extract probed with the surface purified antibodies, there were IgA reactive bands, weakly detected at approximately 60-100 kDa (Figure 5.11, Lane 1). In the lane of S2 extract probed with the surface-purified antibodies, IgA reactive bands were detected at 30 kDa and in the range 80 kDa to >100 kDa (Figure 5.11, Lane 5). Abomasal mucus IgA reactivity against both S1 and S2 antigens was detected, in the range of 20 kDa to >200 kDa, in the lanes probed with non-affinity purified antibodies from previously infected/challenged sheep (Figure 5.11, Lanes 3 and 7, respectively). No abomasal IgA activity was detected in the lanes of S1 and S2 extracts probed with antibodies generated from helminth-naïve sheep (Figure 5.11, Lanes 2 and 6) or the no primary antibody controls (Figure 5.11, Lanes 4 and 7).

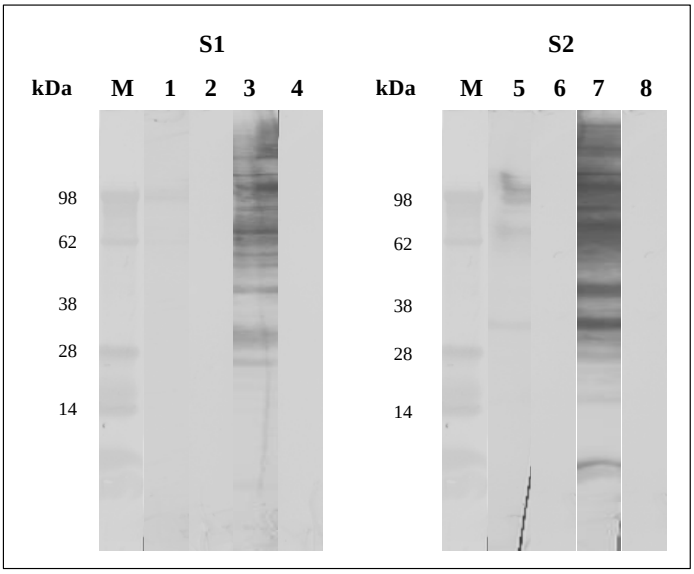

Figure 5.11 Immunoblot of ovine IgA binding to *T. circumcincta* L_3 extracts by probing with purified (affinity-purified against the surface of exsheathed *T. circumcincta* L_3) antibodies from the abomasal mucus obtained from previously infected/challenged sheep.

Lanes 1–4: *T. circumcincta* L_3 S1 extract (5µg/lane). Lanes 5–8: *T. circumcincta* L_3 S2 extract (5µg/lane). Lanes 1 and 5: probed with abomasal antibodies obtained from previously infected/challenged sheep and affinity purified using surface antigens on exsheathed *T. circumcincta* L_3. Lanes 2 and 6: probed with abomasal antibodies obtained from sheep reared in helminth-free conditions and purified against surface antigens on exsheathed *T. circumcincta* L_3. Lanes 3 and 7: probed with abomasal antibodies collected

from sheep trickle-infected with *T. circumcincta* (the abomasa were collected at post mortem 2 days following a bolus challenge of 50,000 L_3). Lanes 4 and 8: no primary antibody controls. All lanes were probed with the appropriate secondary and tertiary antibodies for IgA detection. Lane M depicts standard molecular weight markers given in kDa.

Sodium periodate treatment lead to a decrease in band density but it did not deplete the level of reactivity of the purified IgA to L_3 antigens in S1 (Figure 5.12, Lane 3) and S2 extracts (Figure 5.12, Lane 7).

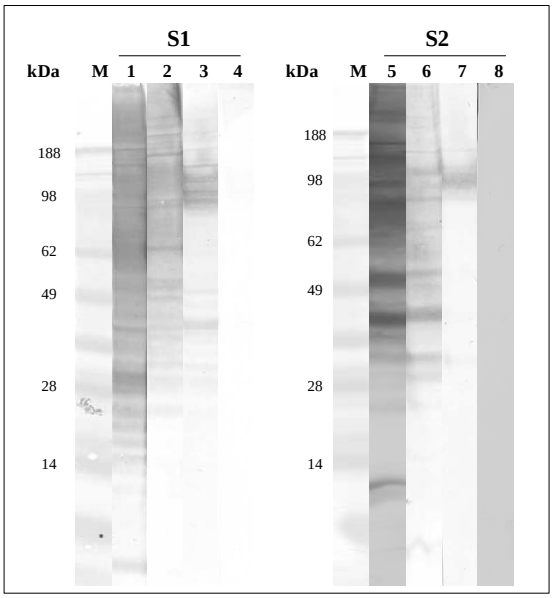

Figure 5.12 Immunoblots demonstrating the effect of sodium periodate treatment of *T. circumcincta* L_3 extracts on their binding to abomasal IgA purified against surface antigens of *T. circumcincta* L_3.

Lanes 1–4: *T. circumcincta* L_3 S1 extract (5µg/lane). Lanes 5–8: *T. circumcincta* L_3 S2 extract (5µg/lane). Lanes 1 and 5: No sodium periodate. Lanes 2, 3, 6 and 7: antigens treated with 50mM sodium periodate prior to antibody probing. Lanes 1, 2, 5 and 6: probed with pool of abomasal mucus from sheep given a trickle infection/bolus challenge. Lanes 3 and 7: probed with abomasal antibodies which were obtained from previously infected/challenged sheep and purified against surface antigens on exsheathed *T. circumcincta* L_3. Lanes 4 and 8: no primary antibody controls. All lanes were incubated with the appropriate secondary and tertiary antibody for detection of IgA binding. Lane M represents molecular weight markers given in kDa.

5.3.6 Biopanning of surface-purified, eluted IgA against a 7-mer phage display library

To determine specific phage enrichment after each round of panning the heptamer phage library against the L_3 surface-purified abomasal IgA, the titre of the amplified phage eluted from each round was determined and quantified as pfu/ml (Table 5.4).

Round	Input phage (pfu/ml)	Amplified phage eluted (pfu/ml)
1	1.0×10^{11}	1.28×10^{9}
2	1.0×10^{11}	1.71×10^{13}
3	1.0×10^{11}	1.63×10^{5}*

Table 5.4 Titres of input phage and amplified phage eluted from each round of biopanning.

A heptamer phage display library was biopanned with abomasal IgA purified against antigens on the surface of exsheathed *T. circumcincta* L_3. * denotes unamplified eluate. Pfu/ml = plaque-forming units per ml.

Following three rounds of screening, 50 bound phage clones were randomly selected and the peptide sequences of the insert regions determined (see Appendix 3 for full list of sequences). The peptide sequences that were highly repeated in the dataset are summarised in Table 5.5.

Peptide	Number of repetitions in dataset	Target-unrelated peptide	Identified in other studies*	Identified in L_3 glycan panning	Bound by "control" antibodies	Clone ID assigned for further experiments
WTPSVRP	3					1
WPTLQWA	5			Yes		2
HAIYPRH	3	Yes	Yes	Yes	Yes	3
QPWPTSI	2					4
SWPQRTN	2					5
GWPKFTK	2					6
ALGIDSG	2					7
KLPGWSG	3					8

Table 5.5 Frequency of repeated peptide sequences from phage clones.

Phage clones were selected through panning a 7-mer phage display library with IgA purified against surface antigens on exsheathed *T. circumcincta* L_3. *Listed on the MimoDB search facility compiled of collated data from phage display library panning experiments

Bioinformatic analysis of the peptide sequences revealed that one of the repeated sequences, HAIYPRH, was classified as a target-unrelated peptide (TUP); this sequence had been identified in the previous panning experiments with L_3 glycan and control antibodies (Section 4.3.2). One of the sequences repeated more than twice in this dataset, WPTLQWA (referred to as clone 2 in current chapter), matched one of the sequences reported in the L_3 glycan antibody panning (section 4.3.2; referred to as Clone 8 in chapter 4).

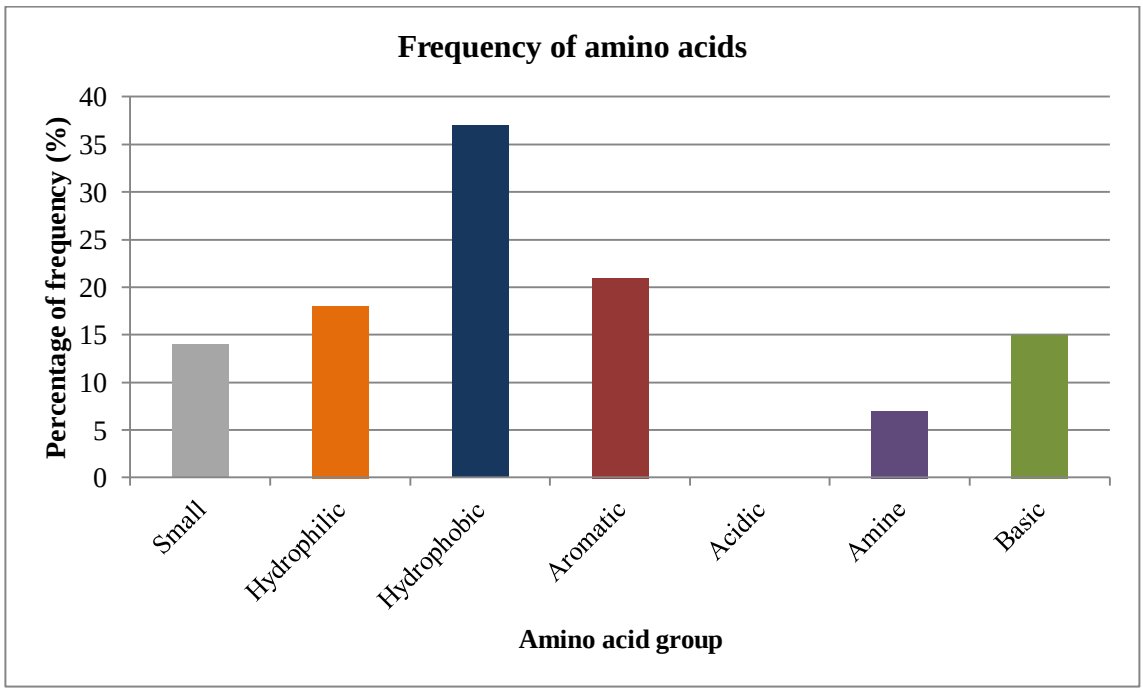

Figure 5.13 Frequency of amino acids in the peptide sequences identified by biopanning a random heptamer phage display library with affinity purified ovine IgA which binds to surface antigens on exsheathed *T. circumcincta* L_3.

Groups of amino acids: Small (Glycine, Alanine); Hydrophilic (Serine, Threonine, Cysteine); Hydrophobic (Valine, Leucine, Isoleucine, Methionine, Proline); Aromatic (Phenylalanie, Tyrosine, Tyrptophan); Acidic (Aspartic acid, Glutamic acid); Amine (Asparagine, Glutamine); Basic (Histidine, Lysine, Arginine). Percentage frequency of amino acid groups was calculated as the total number of repetitions of individual amino acids in the dataset (all amino acids in all bound phage) as a proportion of the total number of amino acids.

Following sequence analysis of the peptide clones, the proportion of the different classes of amino acids in the sequences indicated that there were amino acid residues belonging to the hydrophobic, aromatic and hydrophilic groups (Figure 5.13).

5.3.7 Target specificity of phage clones

Eight of the phage clones selected by biopanning were evaluated by ELISA for their target antibody specificity by comparing the level of background binding with the level of binding to wells coated with antibodies purified against L_3 surface antigens. The results confirmed that the surface purified antibodies bound in a specific manner to all eight phage clones. Clones 1, 2, 3, 5, 6 and 7 demonstrated the highest OD values in the ELISA when mean levels of IgA binding in antibody-coated wells were compared to the uncoated wells (Figure 5.14). Clone 3 bound IgA in the antibodies purified against the surface of exsheathed *T. circumcincta* L_3 (Figure 5.14) and also bound IgA from the abomasum of sheep exposed to a single primary challenge infection of *T. circumcincta* (section 4.3.3), but is a known target unrelated peptide (TUP), so was subsequently removed from further analysis.

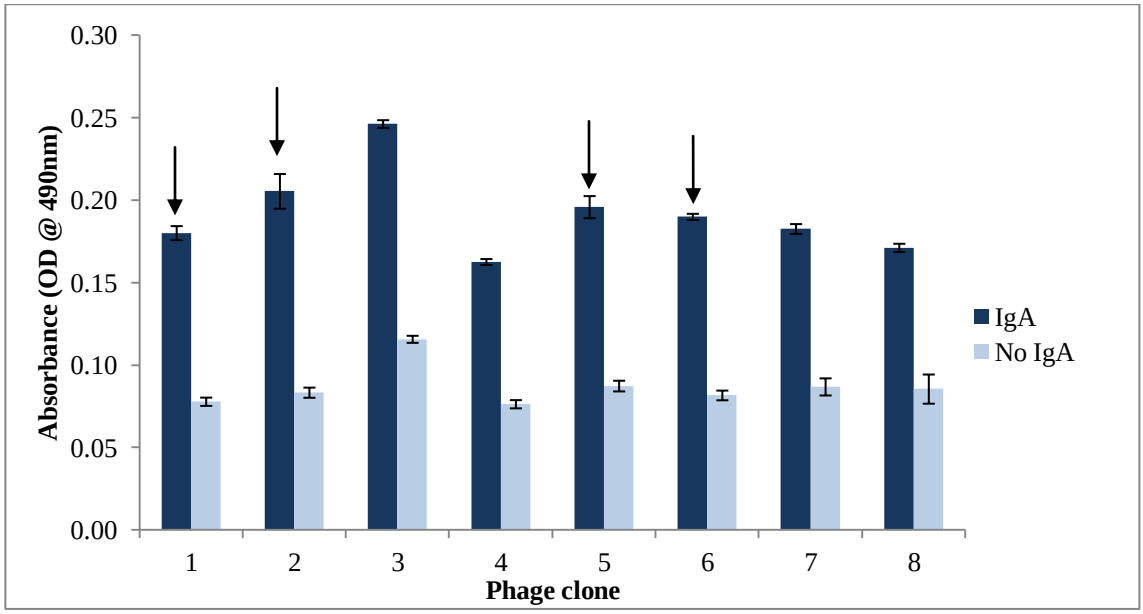

Figure 5.14 Screening ELISA used to investigate the target specificity of eight selected phage clones by comparison of the level of specific binding to abomasal IgA to background.

Arrows denote clones selected for further investigation. Clones 1, 2, 5 and 6 gave the highest OD values in the ELISA when the mean levels of IgA binding in IgA coated wells were compared to those in the uncoated wells. Clone 3 was not selected due to its identification as a target-unrelated peptide. There was an insufficient volume of phage supernatant for both clone 7 and 8 and as a result these were not further investigated.

5.3.8 Phage ELISAs probed with efferent gastric lymph IgA from sheep with differing levels of acquired immunity to *T. circumcincta*

The levels of IgA binding to peptide structures displayed by four of the phage clones identified by biopanning were quantified using gastric lymph obtained from sheep with varying levels of immunity to *T. circumcincta*. Relationships between levels of IgA binding and parasitological and immunological parameters were investigated and are summarised in Table 5.6.

	Nematode burden		Total IgA in lymph		% inhibited larvae	
Clone	**r_s value**	**P value**	**r_s value**	**P value**	**r_s value**	**P value**
1	-0.252	0.246	0.621	0.006*	0.458	0.028*
2	-0.696	<0.001***	0.836	<0.001***	0.595	0.003**
5	-0.646	<0.001***	0.755	<0.01**	0.562	0.005**
6	-0.528	<0.01**	0.814	<0.001***	0.593	0.003**

Table 5.6 Summary of the output from the statistical analysis of the relationships between the levels of gastric lymph IgA binding to four phage clones and parasitological and immune parameters.

'*' and '*' denote significance levels with p-values < 0.05 and <0.01, respectively.The association and relationships were tested by non-parametric analysis with Spearman correlation coefficient. r_s value indicates the strength of the relationship.

For three of the four phage clones investigated, significant negative correlations ($P<0.01$, r_s = -0.528 to -0.696) were observed between the peptide-specific IgA responses in efferent gastric lymph samples and nematode burdens at necropsy (Figure 5.15, Table 5.6). Clone 2 showed the strongest relationship between the level of binding of efferent gastric lymph IgA binding to the peptide and the total nematode burden at necropsy.

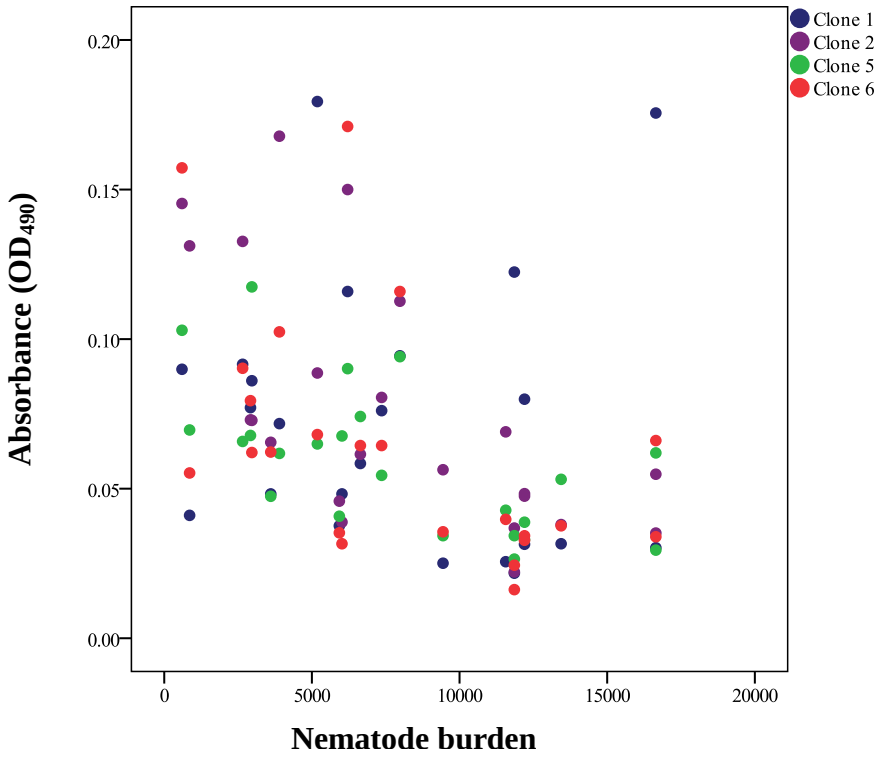

Figure 5.15 Relationship between the levels of gastric lymph IgA binding to four phage clones selected by biopanning and the nematode burden of sheep experimentally infected with *T. circumcincta* L_3.

Analysis of the association was conducted through use of Spearman correlation coefficients. Data points represent the absorbance values from the individual sheep gastric lymph samples (*n*=19), reflecting the level of efferent gastric lymph IgA binding to the phage clone under investigation. The absorbance values were plotted against the total nematode burden for each individual sheep. The efferent gastric lymph was collected from individual sheep ("GL-PI" group) which had been previously infected with *T. circumcincta* L_3 and were subjected to a bolus challenge of 50,000 L_3. The gastric lymph samples used were all from a single time-point of 7 dpc. The worm burden of the "GL-PI" sheep was assessed by counting the number of males, females and inhibited L_4 in a sub-sample of the digests of both the abomasal tissue and contents as published in Halliday *et al*. (2007).

Significant positive correlations ($P<0.05$, $r_s = 0.458$ to 0.595) existed between the level of IgA binding to all the four phage clones and the percentage of inhibited L_4 present at necropsy (Figure 5.16, Table 5.6). Clones 2 and 6 showed the stronger relationships between the level of IgA binding and the percentage of inhibited larvae recorded. There are a lot of data points in which the percentage of inhibited L_4 present were recorded as

0 (Figure 5.16). These are the data pertaining to both the helminth-naïve sheep and the sheep only exposed to a single bolus challenge of 50,000 L_3.

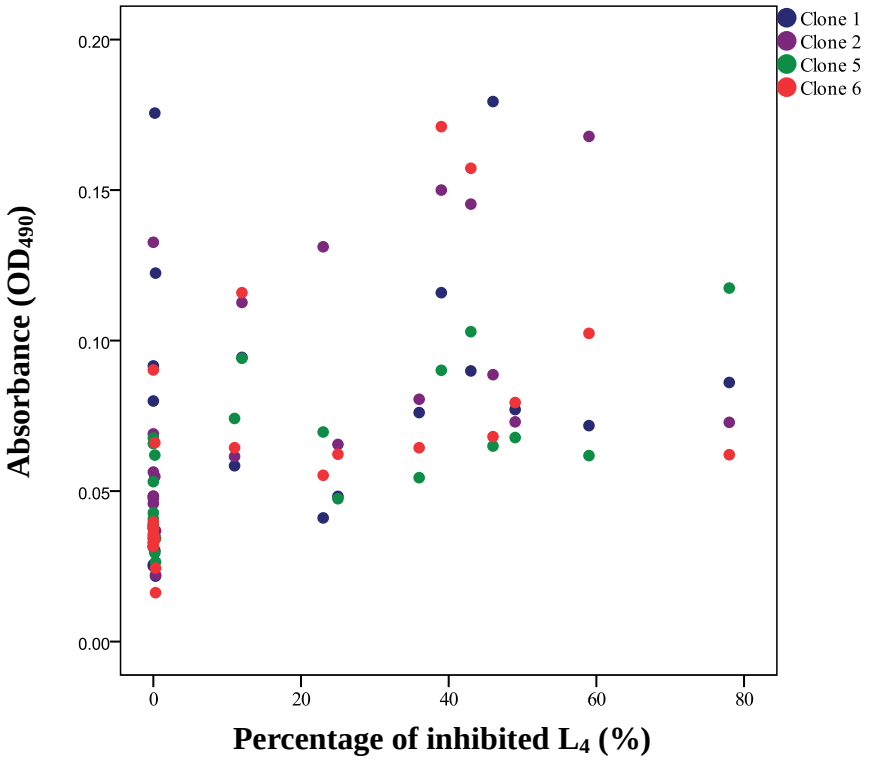

Figure 5.16 Relationship between the levels of gastric lymph IgA binding to four phage clones selected by biopanning a phage display library with L_3 surface-purified ovine antibodies and percentage of inhibited *T. circumcincta* L4s present in the abomasa of sheep experimentally infected with *T. circumcincta* L_3**.**

Analysis of the association was conducted using spearman correlation coefficient. Data points represent the absorbance values from the individual sheep gastric lymph samples (*n*=19), reflecting the level of efferent gastric lymph IgA binding to the phage clone under investigation. The absorbance values were plotted against the percentage of inhibited L_4. The efferent gastric lymph was collected from individual sheep which had been previously infected with *T. circumcincta* L_3 and were subjected to a bolus challenge of 50,000 *T. circumcincta* L_3 ("GL-PI"). The gastric lymph samples used were all from a single time-point of 7 dpc. The percentage of inhibited L_4 present in the abomasum were estimated by directly measuring the length of L_4 in a sub-sample and the percentage calculated from the total worm burden (as detailed in Halliday *et al*., 2007).

Strong positive correlations ($P<0.01$, $r_s = 0.621$ to 0.836) were found between the level of ovine IgA binding specifically to all of the peptide sequences and the total IgA concentration in the efferent gastric lymph at 7 dpc (Figure 5.17, Table 5.6). The relationship between the level of gastric lymph binding to clone 2 and the concentration of total IgA present in the efferent lymph was the strongest (Figure 5.17).

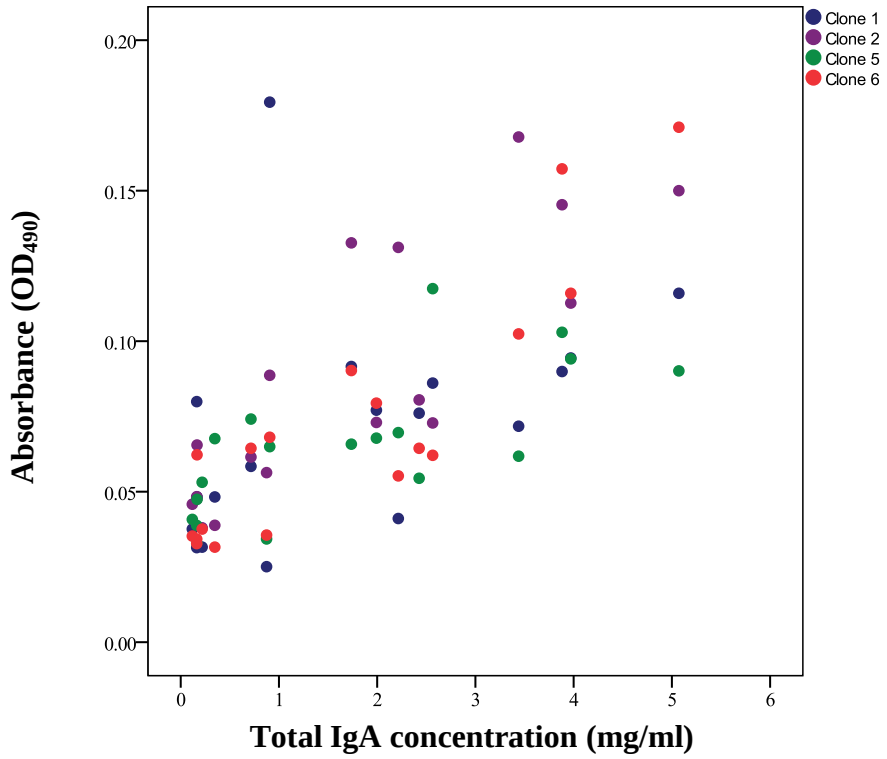

Figure 5.17 Relationship between the levels of gastric lymph IgA binding to four phage clones selected by biopanning a phage display library with L_3 surface-purified ovine antibodies and the total gastric lymph IgA concentration at 7 dpc of sheep previously infected/challenged with *T. circumcincta*.

Analysis of the association was conducted using Spearman correlation coefficient. Data points represent the absorbance values from the individual sheep gastric lymph samples (n=19), reflecting the level of efferent gastric lymph IgA binding to the phage clone under investigation. The absorbance values were plotted against the total IgA concentrations for the individual sheep samples. The efferent gastric lymph was collected from individual sheep which had been previously infected with *T. circumcincta* L_3 and were subjected to a bolus challenge of 50,000 *T. circumcincta* L_3 ("GL-PI"). The gastric lymph samples used were all from a single time-point of 7 dpc. The total IgA concentrations of the efferent gastric lymph of the previously infected/challenged sheep used in this current study were previously published in Halliday *et al.*, (2007).

5.4 Discussion

In the work presented in this chapter, immunofluorescent staining with abomasal mucus, collected from sheep which had been experimentally infected with a *T. circumcincta* trickle infection and bolus challenge regime, revealed that both abomasal mucus IgA and IgG were able to bind native antigens present on the surface of live, intact, exsheathed L_3. When eluted from the nematode larval surface and incubated with a random peptide phage display library to identify peptide sequences which mimic native structural epitopes on the surface of the larvae, specific peptide sequences which bound these affinity-purified antibodies were identified from the library. The immunoreactivity of the peptide structures displayed by four of the phage clones selected by biopanning was also investigated by comparing peptide-specific IgA levels to known correlates of immunity to *T. circumcincta* in sheep with varying levels of immunity (Halliday *et al*., 2007). For three of the four clones subjected to further investigations, positive associations existed between the levels of peptide-specific lymph IgA and both the total IgA concentration in efferent gastric lymph at 7 dpc ($p<0.01$) and the percentage of inhibited L_4 present ($p<0.05$). In addition, significant negative correlations between the levels of peptide-specific lymph IgA and the total *T. circumcincta* burden were also observed ($p<0.01$) for the same three clones.

Work presented in earlier Chapters indicated that mucus antibodies from the abomasum of immune sheep bind an array of antigens in somatic extracts of *T. circumcincta* larval stages. Here, experiments focused specifically on antigens present on the surface of *T. circumcincta* L_3 because previous studies demonstrated a degree of success in inducing protective immunity to *T circumcincta* using antigens purified from the L_3 surface (Wedrychowicz *et al*., 1992; 1995), a phenomenon also demonstrated for other trichostrongylid nematode spp. (Ashman *et al*., 1995; Bowles, Brandon and Meeusen, 1995; Piedrafita *et al*., 2012). Previously exsheathed *T. circumcincta* L_3 were incubated with abomasal mucus from sheep which had received three truncated infections of 30,000 *T. circumcincta* L_3 over 14 days, boosted with 3 separate bolus infections of 30,000 before necropsy 14 days later (Harrison *et al*., 2008). In that study,

there was strong binding of mucus and serum IgG to the surface antigens. However, in the work presented in this study, it was IgA in abomasal mucus which bound to surface antigens. This difference in the isotype stimulated in the immune response could be explained by differences in the trickle infection programme. As in the work presented here, sheep were exposed to a smaller dose of the parasite (*c*. 104,000 L_3) and were continually trickle-infected over a nine-week period compared to exposure of the sheep to a larger dose (*c*. 180,000 L_3) over the shorter four-week period in the work presented by Harrison *et al*. (2008). Also in the latter study, surface antigens were prepared directly from *T. circumcincta* L_3 through a reduction and centrifugation procedure whereas in the work presented in this thesis the aim was to retain the native conformation of the surface antigens so antibodies directly against the surface of exsheathed infective larvae were purified.

Here, antigens present on the surface of exsheathed *T. circumcincta, T. colubriformis* and *H. contrortus* L_3 all bound abomasal mucus IgA and IgG from sheep rendered immune to *T. circumcincta*. This suggests that homologous immunoreactive surface antigens are present in these species. Cross-reactivity of mucus antibodies directed against surface antigens of nematodes has been seen against other truichostrongylid species. Intestinal mucus IgG taken from sheep rendered immune to *T. colubriformis* infection reacted with antigens in larval extracts of both *H. contortus* and *T. circumcincta* (Harrison *et al*., 2003a). Also, the monoclonal antibody specific to a L_3 specific *T colubriformis* carbohydrate larval antigen, *CarLA,* showed cross-reactivity against the surface of *H. contortus* and *T. circumcincta* L_3 (Harrison *et al*., 2003a; 2003b). This raises the possibility that developing a vaccine against surface antigens of *T. circumcincta* L_3 could provide protection against other closely related nematodes.

A surface antigen-specific to exsheathed L_3 of *H. contortus,* Hc-sL_3, has been characterised as a glycoprotein of approximately 70-90 kDa (Ashman *et al*., 1995; Bowles *et al*., 1996; Raleigh *et al*., 1996). Following incubation of exsheathed *H. contortus* L_3 with a rabbit monoclonal antibody specific to Hc-sL_3, in 10-15 min larvae

were observed to have begun to shed the bound anti Hc-sL$_3$ antibodies, and after 30 min of incubation all larvae had shed the antibodies indicating that the antigen is potentially involved in an immune evasion strategy (Ashman *et al*., 1995; Bowles *et al*., 1995). A monoclonal antibody which bound Hc-sL$_3$ was used to probe live *H. contortus* L$_3$ and immunoblots of *H. contortus* and *T. circumcincta* surface extracts (Raleigh *et al*., 1996). In the *T. circumcincta* surface extract blot probed with the Hc-sL$_3$ monoclonal antibody, antibody reactivity was evident at 95-100 kDa (Raleigh *et al*., 1996), and had a similar profile to the immunoreactivity to *T. circumcincta* extracts detected here. Vaccination studies with Hc-sL$_3$ revealed that, when administered with an appropriate adjuvant, it is able to confer protective immunity in sheep against a challenge infection with *H. contortus*, with significant reductions in worm burdens (range, 45-69%) in vaccinated sheep compared to non-vaccinated controls (Jacobs *et al*., 1999; Piedrafita *et al*., 2012). The presence of homologous immunoreactive L$_3$ surface antigens across trichostrongylid species has also been investigated through the use of ASCs generated by the *in vitro* culture of lymph nodes removed from sheep 5 days after administration of a trickle challenge (3,000 *T. circumcincta* L$_3$ per week for nine-weeks) and then a bolus dose of 50,000 *T. circumcincta* L$_3$ (Balic *et al.,* 2003). Parasite extracts of soluble antigens prepared from L$_3$ of *H. contortus*, *Trichostrongylus vitrinus* and *T. circumcincta* were incubated with antibodies secreted by the ASCs and a high molecular weight region of immunoreactivity was identified in each of the three species. The regions of reactivity were, however, slightly different in each. This reiterates the potential ability of antibodies against L$_3$ antigens to cross-react with similar antigens in other nematode species.

The purpose of using surface immunoaffinity purification of antibodies was to enrich the preparations for IgA which reacted with L$_3$ surface antigens under native conditions, i.e. antibodies which reacted with the conformational structures of surface antigens. Following probing of an immunoblot of *T. circumcincta* S1 and S2 antigen preparations with the affinity-purified antibodies, areas of IgA reactivity at 30-40 kDa and 70-100 kDa were detected in both antigen preparations. This pattern of

immunoreactivity is similar to that displayed when *T. circumcincta* L_3 preparations were probed with antibodies derived from ASCs which had been harvested from sheep 5 and 10 dpc following a similar trickle infection regime (Balic *et al*., 2003). However, the IgA reactivity at 30-40 kDa was not as clear here as that presented by Balic *et al*. (2003). One of the possible reasons for this is differences between the antigen preparations used in the studies: here, two antigen extracts were used, a PBS-soluble extract (S1) and a membrane-associated extract (S2), whereas in Balic *et al*. (2003), a whole worm extract was prepared by heating the larvae in SDS buffer.

The purified IgA was then used to pan a 7-mer phage display library and sequence analysis of the clones which bound the IgA showed that one peptide sequence, (WPTLQWA), was also identified using IgA which bound *T. circumcincta* L_3 carbohydrate epitope-containing somatic antigens (Chapter 4). This clone, along with 3 others, was used in ELISAs to assess the relationship between the level of phage clone binding by IgA from sheep rendered immune by experimental infection and correlates of immunity against *T. circumcincta*; worm burden, percentage inhibited L_4 and total IgA concentration in efferent gastric lymph (Halliday *et al*., 2007). Strong positive correlations existed between the total IgA concentration in gastric lymph of sheep rendered immune by experimental infection and binding of heptamer-specific IgA to three phage clones. A positive correlation has been reported between the level of IgA binding to L_4 somatic antigens and the percentage of inhibited *T. circumcinta* L_4 recovered from the ovine abomasum (Beraldi *et al*., 2008; Stear *et al*., 1998; 1999). Here, the levels of gastric lymph IgA binding to three phage clones were strongly positively correlated with the percentage of inhibited L_4 recovered post mortem. The relationships between the level of IgA binding of L_3-surface purified antibodies to phage clones and the known correlates of immunity to *T. circumcincta* are similar to those reported in Chapter 4. These findings suggest that the random peptide sequences expressed by the phage clones selected through panning the library with antibodies specific to antigens on the surface coat of exsheathed *T. circumcincta* L_3 could be mimicking immunogenic structural epitopes on native *T. circumcincta* L_3 antigens. The

selected phage clones may therefore represent valid vaccine candidates if they could be presented to the ovine immune system in an appropriate fashion.

Chapter 6：General Discussion

Gastrointestinal nematode parasites are a major constraint to the sheep farming industry due to economic (mostly production-related) and welfare issues (Molento, 2009). Figures available in 2005 estimated that, collectively, gastrointestinal nematode infections cost the UK sheep farming industry in excess of £84 million per annum (Nieuwhof and Bishop, 2005). In temperate regions, one of the most prevalent gastrointestinal nematodes of small ruminants is *Teladorsagia circumcincta* (Bartley *et al*., 2003). This nematode is most commonly controlled using a combination of pasture management and treatment of animals with anthelmintics. There are five classes of anthelmintics currently licensed in the UK for the control of gastrointestinal nematode infections in small ruminants; however, isolates of *T. circumcincta* resistant to three of these have emerged and resistance is now widespread (Bartley *et al*., 2003; 2004; Sargison *et al*., 2007), including reports of multiple drug class-resistance (Sargison *et al*., 2007). With the emergence of anthelmintic-resistant parasites, this has created an interest in developing vaccines as an alternative means of control. Vaccination is a possible option because protective immunity can be induced against *T. circumcincta* after repeated exposure to the parasite through natural exposure or experimental infection (Smith *et al*., 1983; Stear *et al*., 1997). A number of studies have focused on the immunological basis of host resistance to *T. circumcincta*. The majority of these have been experimental studies, which have been designed to mimic larval challenge on pasture (Beraldi *et al*., 2008). From these, it is evident that a protective immune response can control *T. circumcincta* infection at several stages of its development (Halliday *et al*., 2007; Smith *et al*., 1985). An 'immediate hypersensitivity' reaction has been implicated in the exclusion and expulsion of incoming *T. circumcincta* L_3 in sheep which had been rendered immune through a previous experimental infection regime (Greer *et al*., 2008; Jackson *et al*., 2004; Seaton *et al*., 1989). This 'exclusion/expulsion' response has been seen as early as 2 dpc in sheep exposed to a trickle infection of 10,000 *T. circumcincta* L_3 per week for a 9-week period and subsequently administered with a bolus of 50,000 L_3 (Smith *et al*., 1984). Host effects on larval establishment requires

multiple larval challenges to mediate the immediate hypersensitivity response which is mostly directed against incoming L_3 (Balic *et al*., 2000). The local IgA response in the abomasum has also been demonstrated to be an important component of immunity; for example, negative correlations have been found between L_4 length and total lymph IgA concentrations in experimentally infected 5 month-old lambs (Smith *et al*., 1985) and after natural exposure on pasture in 6-7 month-old lambs (Strain *et al*., 2002). In later studies, immunoblotting experiments demonstrated L_3 and L_4 antigen-specific IgA in the abomasal mucus of immune ewes and ELISA experiments indicated an inverse relationship between 3 dpi ES-specific abomasal IgA levels and worm burden following challenge with 50,000 L_3 *T. circumcincta* (Smith *et al*., 2009). Together, these studies demonstrate that sheep can develop natural immunity to *T. circumcincta* and strengthen the possibility of the development of a vaccine control method.

Historically, the biggest challenges in developing a vaccine against *T. circumcincta* have been the lack of knowledge regarding parasite antigens that stimulate protective immunity (for example, Wedrychowicz *et al.*, 1992a; 1992b; Halliday and Smith, 2011). Vaccine development strategies have involved either targeting molecules from different stages of the parasite's life cycle, including the targeting of products excreted/secreted from worms or exposed on the parasite's surface, using antibody probes or the identification of molecules with homology to other known promising vaccine candidates (Nisbet *et al*., 2010a; Smith *et al*., 2009). In the case of *T. circumcincta*, ES molecules released by L_4 have been targeted because they are thought to be readily accessible to the host immune response, are immunogenic and potentially have important functional roles in parasite survival (Nisbet *et al*., 2009; 2010; Redmond *et al*., 2006; Smith *et al*., 2009). Due to its role in protective immunity against *T. circumcincta*, abomasal mucus IgA has been used to identify a number of possible protective antigens, including cathepsin-F (Tci-CF-1) (Redmond *et al*., 2006; Smith *et al*., 2009), activation-associated secreted protein-1 (Tci-ASP-1) (Nisbet *et al*., 2010a; Smith *et al*., 2009) and L_3-surface-associated antigen (Tci-SAA-1) (Nisbet *et al*., 2009). Recently, recombinant protein versions of eight molecules identified through a

combination of bioinformatic, immunomodulatory and immune-recognition studies were administered to sheep in a single vaccine (Nisbet *et al*., 2013). Vaccination induced a protective immune response to a subsequent challenge infection, with significant reductions in egg output and nematode burdens, in repeated trials, of 58-70% and 56-75%, respectively, in vaccinates compared to control sheep which only received adjuvant (Nisbet *et al*., 2013). Despite this recent success, there has been limited success in the development of recombinant subunit vaccines against other gastrointestinal nematodes of livestock (Ellis *et al*., 2012). Of the potential reasons likely to underlie these failures, the most important may be incorrect protein folding and lack of glycosylation. Vaccination trials with *H. contortus* compared the protective capacity of native cysteine proteases (purified from adult *H. contortus* extracts by affinity for thiol sepharose and cystatin) and a bacterial-expressed recombinant cocktail of three isoforms of the cysteine proteases (Redmond and Knox, 2004; 2006), which had been previously identified through thiol sepharose affinity chromatography of *H. contortus* adult extracts (Knox *et al*., 1995; Knox, Smith and Smith, 1999). Sheep immunised with native cysteine proteases had reductions in faecal egg output and worm burden of 48% and 46%, respectively, following challenge compared to unvaccinated controls (Redmond and Knox, 2004). In contrast, bacterial-expressed versions gave 27% and 38% reductions in faecal egg output and worm burden, respectively (Redmond and Knox, 2004; 2006). The suggested reason behind the failure of the recombinant proteins to reproduce the same level of protection as the native antigen was that some of the recombinant proteins produced by bacterial systems were expressed as insoluble forms and/or lacked appropriate glycosylation (Geldhof *et al*., 2007).

Yeast and eukaryotic expression systems have the capability to glycosylate recombinant proteins but these can differ from nematode glycans. An example of this is from a vaccination trial against *Fasciola hepatica* where two different recombinant protein expression systems were used to produce versions of a *F. hepatica* cathepsin L protease. The protective capacity of a yeast-expressed version and a baculovirus-expressed version of cathepsin L protease was assessed through an immunisation trial in

rats. Briefly, rats were vaccinated with either of the recombinant antigens then subsequently challenged with 200 *F. hepatica* metacercariae (Reszka *et al*., 2005). Following challenge infection, the yeast-expressed version gave a reduction of 18% in fluke burden, whereas the baculovirus version gave a reduction of 52% in fluke burden, compared to unchallenged controls (Reszka *et al*., 2005). Further investigations revealed that the yeast-expressed version was hyper-glycosylated, potentially masking protein/conformational epitopes, whereas the baculovirus-produced version had similar N-glycosylation sites to the native version of the cathepsin L (Reszka *et al*., 2005). This study highlights that although some of the recombinant expression systems have the capability to glycosylate antigens, it may not be in the correct format for antibody binding.

In an attempt to try to introduce the appropriate glycosylation (which is similar to that of the desired parasite antigen) on recombinant antigens, the free-living parasite *Caenorhabditis elegans* has been investigated for this purpose. *C. elegans* glycosylation has high mannose content and complex N-glycans (Cipollo *et al*., 2002). Also, as *C.elegans* is relatively close in evolutionary terms to the parasitic nematodes (Blaxter *et al*., 2000), this suggests it is a suitable vector for expression of recombinant antigens. Promising results have been obtained from the use of transgenic *C. elegans* to produce a recombinant version of a cathepsin L cysteine protease, from *H. contortus* (Murray *et al*., 2007). *C. elegans* was used to express an enzymically active version of cathepsin L and administration of this *C. elegans*-expressed antigen in a vaccination trial showed a significant increase in total serum IgG titres post-challenge with 5,000 *H. contortus* L_3 (Murray *et al.,* 2007). Analsysis of the glycosylation patterns of the native cathepsin L antigens and the *C. elegans*-expressed version revealed that both shared the same N-linked glycosylation site (Murray *et al*., 2007).

The overall aim of the work presented in this thesis was to address these issues with recombinant antigen production by employing phage display libraries to identify peptide sequences that mimic the structure of native antigen epitopes in both somatic and surface

extract preparations of *T. circumcincta* L_3. In doing this, a number of techniques were employed;

1) immuno-affinity purification of L_3 antigens under native conditions followed by proteomic/bioinformatic identification of the purified material
2) affinity purification of local antibody probes against L_3 somatic and surface extracts
3) biopanning of the purified antibodies against random peptide phage display libraries.

At the outset, investigations of extracts of *T. circumcincta,* probed with abomasal mucus and gastric lymph generated from sheep which had been previously exposed to the parasite, were used to determine if carbohydrate moieties on larval antigens have a potential role in antibody binding. The results (presented in Chapter 2) suggested that a proportion (approximately 25%) of local IgA reactivity was directed against glycans found on high molecular weight antigens. The presence of glycans in parasite extracts and their capability for modulation of the host immune response is well described (Hokke and Deelder, 2001; Schallig and Leeuwen, 1996; Tawill *et al*., 2004; Vervelde *et al*., 2003). In this thesis, the presence of immunoreactive glycans on parasite antigens was investigated through chemical disruption of their structure. Glycan arrays have been developed that contain hundreds of defined biologically-important glycans (Aranzamendi *et al*., 2011; Blixt *et al*., 2004). The arrays can be screened with antibodies to reveal the profile of glycans bound to the antibodies. One of the first studies to investigate the application of glycan arrays in parasite vaccine development involved interrogating an array containing 264 glycan structures with serum from sheep that were immune to challenge infection following vaccination with *H. contortus* adult ES products (Van Stijn *et al*., 2010; Vervelde *et al*., 2003). This approach identified a novel immunogenic glycan epitope, Galα1-3GalNAc-R, present on *H. contortus* adult ES products (Van Stijn *et al*., 2010). Collectively, these studies show that glycans can be immunogenic. These results from this current study highlight that the role of glycans in the immune response directed against *T. circumcincta* warrants further investigation in order to identify the specific glycan moieties.

The work described in Chapter 2 also demonstrated, for the first time with L_3 antigens, that using efferent gastric lymph from two individual trickle-infected/bolus-challenged sheep as local antibody probes, there was a clear anamnestic IgA response to the antigens after the administration of an L_3 bolus challenge. Previous studies have shown that a local anamnestic IgA response to L_4 antigens was evident as early as 5 dpc in gastric lymph obtained from trickle-infected/bolus-challenged sheep (Halliday *et al*., 2007; Smith *et al*., 1983; 1984). The results here using the same local antibody probes indicated that L_3 antigen-specific IgA levels began to increase at an earlier time-point of 4 dpc. However, there are limitations to this result as gastric lymph samples across the time-frame of -2 to 10 dpc were only available from two sheep. To strengthen this finding, one possible option is to repeat the model infection, focusing on a more defined time period, for example -1 to 5 dpc, to extend the number of sheep tested.

T. circumcincta-L_4 somatic antigen-specific IgA levels in ovine abomasal mucosa have been shown to have an inverse relationship with the length of L_4 recovered from the abomasum at post mortem (Stear *et al*., 1998; 1999; 2004; Strain and Stear, 1999). In the study from which the gastric lymph samples used throughout this thesis were obtained (Halliday *et al*., 2007), the authors concluded that the peak in IgA concentration in lymph occurred too late to be associated with the parasite loss and arrested development that was seen by 5 dpc. Here, we report a strong positive association between *T. circumcincta* L_3 extract-specific IgA levels in the gastric lymph samples used in that study (Halliday *et al*., 2007) and the percentage of inhibited L_4 in the abomasa of the sheep from which the lymph was derived. This raises the possibility that although the peak IgA levels against L_4-specific antigens occur later, i.e. 6-10 dpc, than the onset of inhibition of development at 5 dpc (Halliday *et al*., 2007), IgA specific to L_3 antigens could be triggering the effect. Therefore, L_3-specific IgA levels could contribute to the inhibition of development of L_4 in the abomasum.

In the work presented in Chapter 3 of this thesis, immunoaffinity chromatography was used to purify IgA-reactive L_3 somatic antigens under native conditions to retain epitope structure. A proteomics approach was employed to identify

T. circumcincta proteins in the IgA-affinity purified L_3 antigens. The proteomic analysis identified seven antigenic molecules purified by IgA which were of interest as potential vaccine candidates; paramyosin, activation-associated secreted protein (ASP-2), fatty acid/retinol binding protein (FAR), superoxide dismutase, glutathione-S-transferase (GST), galectins and venom-like allergen protein (VAL). Paramyosin is a component of muscular filaments and has been found to be immunogenic in a range of parasitic nematodes: *Brugia malayi* (Li *et al*., 1999), *D. viviparus* (Strube *et al*., 2009), *T. colubriformis* (Kiel *et al*., 2007) and *T. spiralis* (Wei *et al*., 2010). ASPs are nematode-specific members of a protein family and thought to have a key role in the establishment of parasites in the host (Hawdon *et al*., 1999). For the first time, ASP-2 was identified in the L_3 stages as previously only ASP-1 has been described from *T. circumcincta* L_4 ES products (Nisbet *et al*., 2010a; Smith *et al*., 2009). These molecules are key vaccine candidates against hookworms in humans and are thought to be important in the establishment of parasites in the host (Hawdon *et al*., 1999; Tawe *et al*., 2000). Another group of nematode-specific molecules are represented by the FARs; these are proposed to play a role in the sequestration of retinol/vitamin A which is essential for the maintenance of parasite growth (Basavaraju *et al*., 2003; Wolff and Scott, 1995). Sequestration of retinol/vitamin A by the parasite from the hosts' reservoirs could thereby promote parasite survival. The two detoxifying enzymes identified here, superoxide dismutase and GST, have previously been identified in the L_3 stages of a number of gastrointestinal nematodes (Hadas and Stankiewicz, 1998; Knox and Jones, 1992) and may have a function in counteracting oxidative stress associated with the mucosal inflammatory response during parasite invasion (LoVerde, 1998). As the antioxidant activity of superoxide dismutase has been noted to be higher in L_3 than in adult stages of a number of gastrointestinal parasitic nematode species, including *T. circumcincta* (Hadas and Stankiewicz, 1998; Knox and Jones, 1992), this suggests that they may be ideal vaccine candidates targeted towards L_3. Galectins represent a lectin family with high affinity for β-galactosidase and have been identified in a number of helminths: *H. contortus* (Greenhalgh *et al*., 2000; Newlands *et al*., 1999), *T. circumcinct*a and *T. colubriformis* (Greenhalgh *et al*., 1999). The roles of galectins in

parasitic nematodes are not fully understood, but there are suggestions that they modulate the immune response through mimicking host galectins to evade parasite binding (Vasta, 2009; Young and Meeusen, 2004). It is possible that some of these antigens could be incorporated into future prototype sub-unit vaccines for *T. circumcincta*.

Developing recombinant sub-unit vaccines against gastrointestinal nematodes is proving to be a difficult task, due to factors including complexity of parasite antigens, difficulty expressing recombinant versions which are homologous to native antigens, antigenic variation across different life-cycle stages and difficulty in stimulating local immune responses i.e. at the gut mucosa (Knox and Smith, 2001). As discussed earlier, one of the most pressing issues in nematode vaccine development is the importance of structural epitopes of protective antigens. Identification of structural, or conformational, epitopes is not straightforward because they are formed by post-translational modifications (for example, glycans) and/or amino acid residues on separate areas of the antigen which, when brought into close proximity during tertiary protein folding, form a three-dimensional epitope (Goldsby *et al*., 2003). In this thesis, antibody panning of combinatorial random peptide phage display libraries was used in an attempt to target and identify structures that might mimic potential conformational epitopes of *T. circumcincta* L_3 antigens. The first pool of antibodies used in panning was affinity-purified using the high molecular weight glycan moieties associated with L_3 somatic antigens, while the second pool was affinity-purified directly against the surface of live, exsheathed *T. circumcincta* L_3. The first of these approaches used immunoblots, which, while retaining glycan structures intact, may have resulted in the loss of protein-folding-dependent conformational epitopes due to denaturation in the electrophoresis steps. In order to address this, the second approach was developed to retain the 'native' conformation of antigenic epitopes on the surface of the live, exsheathed *T. circumcincta* L_3. IgA antibodies, purified by both of these methods, bound phage-displayed heptameric peptides in the panning experiments and sequence analysis of the heptamers revealed that the most frequently repeated clone in both data sets was the peptide,

WPTLQWA. This clone, to our knowledge, has not been identified in previous phage display biopanning studies and could represent a valid peptide sequence for inclusion in a novel epitope-based vaccine. In addition to WPTLQWA, five IgA-binding phage clones expressing heptameric peptides, potentially representing epitopes of L_3 somatic and surface antigens, were used in ELISA experiments to assess the relationship between IgA:heptamer binding and correlates of immunity in sheep. IgA in the gastric lymph from these sheep has previously been shown to bind L_4 somatic antigens (Halliday *et al*., 2007) and work presented here showed that IgA in these samples also binds to L_3 antigens and to the selected phage clones expressing heptameric peptides potentially representing epitopes of *T. circumcincta* L_3 somatic and surface antigens (Figure 4.8 and Figures 5.15-5.17). Four quantitative parameters were analysed to establish the relationship between IgA:hepatmer binding and indicators of immunity. These were;

1) total nematode burden,
2) percentage of inhibited L_4,
3) total IgA concentration in efferent gastric lymph, and
4) the lymphoblast response.

Each of these measures has previously been associated with development of protective immunity in *T. circumcincta* infected sheep (Halliday *et al*., 2007). The efferent gastric lymph samples from trickle-infected/bolus-challenged and primary bolus-infected sheep used in these experiments were from one single time-point, 7 dpc. This time-point corresponded to the peak in total IgA measured in these samples (Halliday *et al*., 2007). Here, significant positive correlations between total IgA concentration at 7 dpc and binding of heptamer-specific IgA to six phage clones were evident (Figure 4.13 and Figure 5.17). Levels of gastric lymph IgA specific to these six phage clones were also significantly positively correlated with percentage of inhibited L_4. In contrast, no correlations were found between the level of gastric lymph IgA specific to the phage clones and the lymphoblast response. Collectively, these correlations between the known immune parameters and the level of peptide/phage clone binding strengthens the possibility that the structures formed by the display of the peptides on the phage surface

mimic those of structural immunoreactive epitopes found on native *T. circumcincta* L_3 antigens and warrants further investigation into their potential as vaccine candidates for teladorsagiosis.

If a vaccine trial were to be conducted with the peptide sequences, careful consideration would need to be used in the design of the formulation, whether it be construction of the peptide sequences synthetically or using the phage vector to directly express the peptide sequence(s). Administration of the peptides as surface-displayed antigens on the phage vector has previously been the preferred method of presentation, for example in a vaccine trial assessing the protective capacity of peptide mimics of cathepsin L epitopes to protect against fasciolosis in sheep (Villa-Mancera *et al*., 2008; 2010). This formulation, when administered to the sheep as a subcutaneous injection with no adjuvant, gave a significant reduction in mean fluke burdens (range 34-48%) compared to unvaccinated control sheep following an experimental oral challenge with 300 metacercariae (Villa-Mancera *et al*., 2008; 2010). Given the complexity of the host:parasite interaction between sheep and *T. circumcincta*, it is likely that a multi-component vaccine will be required (Nisbet *et al*., 2013), so a cocktail of the peptide sequences identified in this thesis may be preferable to a single peptide for protection against re-infection with the parasite. If a cocktail of phage-displayed peptides were used in a vaccine, it may still be more cost effective and commercially attractive to produce, in comparison to the production of 8 recombinant proteins, due to the different vectors and expression systems required. Also, the phage-displayed peptides could be administered with the vaccine currently under development (Nisbet *et al*., 2013). Ovine abomasal mucus antibodies specific to each one of the 8 vaccine components could be screened against a phage display library in order to identify peptide sequences which represent structural epitopes on the recombinant versions of the cocktail vaccine antigens. Recently, phage-based products have been approved for use in food safety by the US Food and Drug Administration, as a cocktail of several phage clones, termed ListShield™, targeting *Listeria monocytogenes* contaminants in meat and poultry products are now licensed for commerical use in the USA (Hunter, 2011). The large-

scale production of phage-displayed peptides on a large-scale through the cloning and purification protocols would be relatively simple and cost effective (Clark and March, 2006). These trials would need to evaluate what effect vaccinating with the phage display peptides had on worm burden, egg output, inhibition of development and specificity of the immune response generated.

In conclusion, the work presented in this thesis has targeted the conformational epitopes on *T. circumcincta* L_3 antigens through two avenues; (i) purification of 'native' L_3 antigens retaining the structural epitopes and (ii) identification of phage-displayed peptide sequences with the potential to mimic structural epitopes. Affinity purified L_3-antigen-specific IgA levels in sheep with varying levels of immunity to *T. circumcincta* were positively correlated with both the total IgA concentration in efferent gastric lymph after parasite challenge and with the percentage of inhibited L_4 larvae present in the gastric glands of the immune hosts. In contrast, there was a negative correlation between the level of affinity-purified L_3 antigen-specific IgA and total *T. circumcincta* burden. Proteomic analysis of the IgA-affinity purified L_3 extract identified a number of proteins which are potential vaccine candidates; paramyosin, superoxide dismutase, galectin, activation-associated secreted proteins and FARs. Due to the demonstrated immunoreactivity and the fact that some of the proteins are components of a vaccine under development, it strongly suggests that they could be components of a novel vaccine targeted towards *T. circumcincta* L_3 to prevent their establishment in the ovine abomasum.

As a first step towards the development of a novel vaccine based on IgA-binding-peptide mimics of native structural epitopes, phage display libraries were used to screen antibodies, from sheep rendered immune to *T. circumcincta* by experimental infection. These antibodies were affinity-purified before use and specifically bound *T. circumcincta* L_3 glycans or, alternatively, surface antigens on exsheathed *T. circumcincta* L_3. Six peptide sequences were identified and positive correlations existed between peptide-specific IgA levels and both the total IgA concentration in efferent gastric lymph after parasite challenge and the percentage of inhibited L_4 present.

Collectively, this reveals the potential protective capacity of the phage clones selected for affinity to IgA, purified against somatic and surface L_3 antigens, and shows that this study has identified peptide sequences which mimic conformational epitopes on *T. circumcincta* L_3 antigens. In future, a vaccine trial would be required to determine if the peptide sequences are in fact mimicking protective epitopes on L_3 antigens.

Appendix 1: General solutions and buffers

Nematode culture medium
500ml of RPMI liquid culture medium, 1% (v/v) D-glucose, 2mM glutamine, 100 IU/ml penicillin, 100mg/ml streptomycin, 125 mg/ml gentamycin , 25mg/ml amphotericin B. Stored at 4°C until required.

TNTT (for 1 litre)
10mM Tris, 0.5 M Sodium chloride, 0.05% (v/v) Tween-20, 0.01% (w.v) Thiomersal pH 7.4 adjusted with HCl.

LB broth
10g Bacto-tryptone, 5g Bacto-yeast,5g sodium chloride, dissolved in 1 litre of dH_2O .

LB agar plates
15g bacto-agar added to 1 litre of LB medium, autoclaved at 121ºC for 15 min.

Phosphate buffered saline (PBS)
Stock solution (10x) prepared with 80g sodium chloride, 2g potassium chloride, 11.5g di-sodium hydrogen orthophosphate and 2g potassium di-hydrogen orthophosphate in 1 litre of dH_2O. A working solution was prepared by diluting 1:10 in dH_2O.

IPTG/Xgal stock [conc: 50mg/ml and 40mg/ml]
800µl of Xgal [stock concentration 50mg/ml in dimethylformamide (DMF)] dissolved into 200µl DMF, 50mg IPTG into 1 ml of Xgal/DMF solution.

LB/IPTG/Xgal plates
To 1 litre of LB agar, 1 ml of IPTG/Xgal stock was added and poured into plates.

Top Agar
10g Bacto-tryptone, 5g yeast extract, 5g sodium chloride, 7g Bacto-agar – made up to 1 litre with water, autoclaved at 121ºC for 15 min.

Tetracycline stock (suspension)
Dissolve 20 mg tetracycline dissolved in 500µl dH_20: 500µl ethanol.

LB+ Tetracycline plates
To 1 litre of LB agar 1 ml of Tetracycline stock added and poured into plates.

TBS
50mM Tris, 150mM sodium chloride, pH adjusted to 7.5 with HCl.

PEG/NaCl

20% (w/v) polyethylene glycol-8000 dissolved in 100ml dH_20. Add 14.6g sodium chloride (2.5M). Autoclaved at 121ºC for 15 min.

Iodide buffer

10mM Tris-HCl, 1mM EDTA, 4 M sodium iodide, pH adjusted to 8 with HCl.

Appendix 2: Proteins identified by analysis of IgA-reactive antigens

Protein ID	Organism	Accession number	Peptides	MW	MOWSE	Proposed function
3_58	*Teladorsagia circumcincta*		3	70.1	144.4	Unknown
14-3-3	*Caenorhabditis remanei*	AAY52455	5	25.8	297.1	Metabolism (Amino acid)
14-3-3 like protein	*Caenorhabditis elegans*	Q20655	7	56.9	372.9	Metabolism (Amino acid)
14-3-3 protein	*Angiostrongylus cantonensis*	AEK21299	3	45.8	160.9	Metabolism (Amino acid)
14-3-3-like	*Ascaris suum*	ADY48800	5	23.8	354.9	Metabolism (Amino acid)
3_75	*Teladorsagia circumcincta*		3	26.8	166.1	Unknown
ASP-2	*N. americanus*	AAP41952	13	26.8	166.1	Developmental (Reproduction/Transport)
Alpha-2-macroglobulin domain containing protein	*Haemonchus contortus*	CDJ90107	3	82.9	116.2	Enzymatic (Protease inhibitor)
Alpha-macroglobulin complement component	*Brugia malayi*	XP_001896078	3	44.3	109.9	Enzymatic (Protease inhibitor)
Annexin	*Caenorhabditis elegans*	Q27864	2	49.5	76.1	Cellular (Transport)
Aspartyl protease inhibitor	*Ostertagia ostertagi*	Q95PP1	2	36.9	75.9	Proteolytic enzyme (Aspartyl protease inhibitor)
ASP-like protein	*Cooperia punctata*	AAK35199	2	125.9	88.7	Developmental (Reproduction/Transport)
Beta-D-galactosidase	*Brugia malayi*	AAA27859	8	66.3	367.2	Metabolism (Carbohydrate)
Calponin	*Caenorhabditis briggsae*	XP_002639352	2	21.7	116.9	Cytoskeletal protein

Calponin	*Caenorhabditis elegans*	O01542	5	43.2	201.5	Cytoskeletal protein
Calponin	*Haemonchus contortus*	CDJ87225	3	15.8	138.9	Cytoskeletal protein
CBN-CUTL-23	*Caernorhabditis brenneri*	EGT39830	2	37.5	85	Cytoskeletal protein
CBR-PAR-5	*Caernorhabditis briggsae*	XP_002633409	2	11.7	109.6	Developmental (Reproduction/Growth)
CBR-UNC-15	*Caenorhabditis remanei*	CB015261	5	76.9	288.7	Cytoskeletal protein
CD109 antigen	*Ascaris suum*	ADY40184	2	137.5	41.2	Unknown
CRE-ORA-1	*Caernorhabditis remanei*	XP_003108375	6	49.5	319.2	Unknown
C-type single domain activation ASP3 precursor	*Ostertagia ostertagi*	CAO00416	2	46	100.0	Developmental (Reproduction/Transport)
Cyclophilin	*Caenorhabditis briggsae*	XP_002638373	2	22.7	60.0	Developmental (Cuticle synthesis/protein folding)
Cyclophilin-type peptidyl-prolyl cis-trans isomerase-15	*Brugia malayi*	XP_001896264	2	47.5	120.4	Developmental (Cuticle synthesis/protein folding)
Cysteine protease inhibitor	*Heligmosomoides polygyrus*	AGA95986	3	75.8	134.1	Proteolytic enzymes (Cysteine protease inhibitor)
Cysteine-rich secretory (venom allergen like)	*Caenorhabditis brenneri*	EGT59294	9	66.6	441.1	Developmental (Reproduction/Transport)
Cytochrome C	*Ascaris suum*	P92504	4	51.9	294.9	Metabolism (Electron transport)
EGF domain containing protein	*Haemonchus contortus*	CDJ80140	3	47.6	116.0	Unknown
Excretory/secretory antigen	*Ostertagia ostertagi*	CAA86821	7	46	513.8	Unknown

Fatty acid binding protein	*Caenorhabditis elegans*	Q20224	4	54.1	172.8	Metabolism (Lipid binding/ Electron transport)
Fatty acid binding protein	*Haemonchus contortus*	CDJ96356	4	12.4	272.8	Metabolism (Lipid binding /Electron transport)
Fatty acid/retinol binding protein	*Caenorhabditis elegans*	P34382	2	49.2	121.6	Metabolism (Lipid binding /Electron transport)
Ferritin	*Caernorhabditis elegans*	CE20622	5	76.5	285.4	Gut specific (Iron transport)
Fructose bisphosphate aldolase	*Caenorhabditis briggsae*	EGT52826	2	38.9	183	Metabolism (Amino acid)
Fructose bisphosphate aldolase	*Caernorhabditis brenneri*	EGT52826	2	48.7	154.1	Metabolism (Amino acid)
Fructose bisphosphate aldolase	*Caernorhabditis elegans*	P46563	4	57.1	268.1	Metabolism (Amino acid)
Fructose bisphosphate aldolase	*Teladorsagia circumcincta*	CB037380	3	57.1	190.5	Metabolism (Amino acid)
Fructose-1,6-bisphosphate aldolase	*Caenorhabditis remanei*	XP_003110723	4	56.3	150.7	Metabolism (Amino acid)
Fructose-bisphosphate aldolase 2	*Ascaris suum*	ADY45824	2	74.6	83.6	Metabolism (Amino acid)
Galectin	*Haemonchus contortus*	AAF63405	6	99.1	328.0	Metabolism (Carbohydrate binding)
Galectin	*Ostertagia circumcincta*	U67147	9	97.8	501	Metabolism (Carbohydrate binding)
Galectin 1	*Teladorsagia circumcincta*	AAD39095	9	91.4	533.1	Metabolism (Carbohydrate binding)
Galectin 3a	*Haemonchus contortus*	AAF63405	3	54.2	113	Metabolism (Carbohydrate binding)
Galectin 3b	*Haemonchus contortus*	AAF63406	3	205.6	130.8	Metabolism (Carbohydrate binding)
Galectin 4	*Angiostrongylus cantonensis*	AEK98126	4	84.8	217.8	Metabolism (Carbohydrate binding)

Galectin 4	*Caenorhabditis elegans*	Q18625	9	58.6	491.9	Metabolism (Carbohydrate binding)
Galectin 5	*Angiostrongylus cantonensis*	AEK98127	7	56.2	296.4	Metabolism (Carbohydrate binding)
Galectin 8	*Caenorhabditis elegans*	Q0910	2	59.8	49.4	Metabolism (Carbohydrate binding)
Globin like ES protein F6	*Ostertagia ostertagi*	Q8WQ13	5	38	310.6	Unknown
Glutathione S transferase	*Haemonchus contortus*	AAF81283	9	64.1	614.7	Metabolism (Detoxification)
Glutathione S transferase	*Heligmosomoides polygyrus*	AAF36480	13	56.6	854.0	Metabolism (Detoxification)
Glutathione S-transferase	*Necator americanus*	2ON5_A	3	14.1	108.8	Metabolism (Detoxification)
Glycoprotein	*Haemonchus contortus*	CDJ97733	4	187.0	360.6	Unknown
Hypothetical protein	*Caenorhabditis elegans*	F57H12.3	2	48.6	45.2	Unknown
Hypothetical protein	*Caenorhabditis elegans*	CB038581	4	50.9	161.2	Unknown
Hypothetical protein	*Caenorhabditis elegans*	T27561	4	50.3	162.4	Unknown
Hypothetical protein	*Caenorhabditis elegans*	F57H12.3	4	48.6	267.4	Unknown
Hypothetical protein	*Caenorhabditis elegans*	Q23378	3	44.9	136	Unknown
Hypothetical protein	*Caenorhabditis elegans*	Q23642	5	33.3	205.8	Unknown
Hypothetical protein	*Caenorhabditis elegans*	Q10121	3	81.3	130.3	Unknown
Hypothetical protein	*Caenorhabditis elegans*	Q9U315	3	36.1	80.3	Unknown

Hypothetical protein	*Caernorhabditis elegans*	Q9XW37	4	50.6	210.5	Unknown
Hypothetical protein	*Caernorhabditis elegans*	Q23378	3	44.9	141.1	Unknown
Hypothetical protein	*Caernorhabditis elegans*	CAB03140	3	48.1	81.2	Unknown
Hypothetical protein	*Ostertagia ostertagi*	O16784	2	42	140.9	Unknown
Hypothetical protein CAEBREN_10926	*Caenorhabditis brenneri*	EGT55574	5	29.8	328.7	Unknown
Hypothetical protein CAEBREN_13358	*Carnohabditis brenneri*	EGT42038	6	49.6	297.4	Unknown
Hypothetical protein CAEBREN_14566	*Caenorhabditis brenneri*	EGT32585	3	57.1	202.2	Unknown
Hypothetical protein CAEBREN_19973	*Caenorhabditis brenneri*	EGT55713	3	48.4	219.1	Unknown
Hypothetical protein CAEBREN_21511	*Caenorhabditis brenneri*	EGT30028	5	28	273.5	Unknown
Hypothetical protein CAEBREN_21522	*Caenorhabditis brenneri*	EGT30028	9	44.5	265.5	Unknown
Hypothetical protein CAEBREN_22515	*Caenorhabditis brenneri*	EGT50974	6	70.8	297.4	Unknown
Hypothetical protein CAEBREN_23086	*Caenorhabditis brenneri*	EGT45681	2	76.2	76.3	Unknown
Hypothetical protein CAEBREN_26356	*Caenorhabditis brenneri*	EGT57708	2	96.2	95.4	Unknown
Hypothetical protein CAEBREN-16381	*Caernorhabditis brenneri*	EGT30042	2	221.3	48.2	Unknown
Hypothetical protein CBG_18749 CAP36143	*Caenorhabditis briggsae AF16*	CAP36143	3	69.2	152.7	Unknown
Hypothetical protein CBG01374	*Caenorhabditis briggsae*	XP_002636124	2	25.2	85.1	Unknown

Hypothetical protein CBG09301	*Caenorhabditis briggsae*	XP_002636847	5	39.4	194	Unknown
Hypothetical protein CBG13806	*Caenorhabditis briggsae*	XP_002622549	2	75.8	105.4	Unknown
Hypothetical protein CBG22550	*Caenorhabditis briggsae*	XP_002634948	2	19.9	140.9	Unknown
Hypothetical protein CRE_18937	*Caenorhabditis remanei*	XP_003115303	3	61.3	154.4	Unknown
Hypothetical protein CRE_21222	*Caernorhabditis remanei*	XP_003105218	2	111.5	44.8	Unknown
Hypothetical protein CRE_29604 NDK	*Caenorhabditis remanei*	XP_003112004	12	39.2	621.0	Unknown
Hypothetical protein CRE_29767	*Caenorhabditis remanei*	XP_003112257	2	59.6	86.3	Unknown
Hypothetical protein CRE_29767	*Caernorhabditis remanei*	XP_003112257	2	34.1	137.2	Unknown
Hypothetical protein F55H12.4	*Caenorhabditis elegans*	NP_492398	2	49.5	86.3	Unknown
Hypothetical protein O01462	*Caenorhabditis elegans*	CB039019	2	54.8	42.8	Unknown
Hypothetical protein Y105C5B.5	*Caenorhabditis elegans*	NP_502894	8	39.8	316.5	Unknown
Hypothetical protein	*Caernorhabditis elegans*	CAB03140	2	48.1	86.3	Unknown
Intermediate filament protein ifa-1	*Ascaris suum*	ADY43340	2	42.5	111.8	Cytoskeletal protein
L3B25	*Teladorsagia circumcincta*	AAM45145	7	68.6	371.7	Unknown
Lactoylglutathione lyase	*Brugia malayi*	XP_001895191	2	79.7	75.3	Unknown
Major epididymal secretory protein heh-1	*Ascaris suum*	ADY47453	2	14.1	113.6	Metabolism (Lipid binding)

Major epididymal secretory protein HEH-1	*Caenorhabditis remanei*	XP_003099554	6	59	393.4	Metabolism (Lipid binding)
Myosin	*Caenorhabditis briggsae*	CAP36983	12	159.6	733.4	Cytoskeletal protein
Myosin heavy chain	*Haemonchus contortus*	ABC40752	2	265.7	97.6	Cytoskeletal protein
Nippocystatin	*Nippostrongylus brasiliensis*	AB050883	2	47.5	109.8	Proteolytic enzymes (Cysteine protease inhibitor)
Nucleoside diphosphate kinase	*Caenorhabditis elegans*	NP_492761	6	48.9	390.9	Metabolism (ATP synthesis/Electron transport)
Onchocerca related antigen	*Caenorhabditis briggsae*	CAP35226	3	60.8	63.4	Unknown
Onchocerca related antigen	*Caenorhabditis elegans*	CB038581/O45098	8	50.9	497.8	Unknown
ORA-1 protein	*Caenorhabditis briggsae*	CAP35226.2	6	33.7	274.8	Unknown
Paramyosin	*Ancylostoma caninum*	ABC86903	6	101	502.9	Cytoskeletal protein
Paramyosin	*Dictyocaulus immitis*	P13392	7	66.3	447.8	Cytoskeletal protein
Paramyosin	*Dictyocaulus viviparus*	ABO07440	8	100.8	598.4	Cytoskeletal protein
Paramyosin	*Haemonchus contortus*	CB016022	6	113.1	365.4	Cytoskeletal protein
Peptidyl prolyl cis-trans isomerase 5	*Ostertagia ostertagi*	P52013	3	55	219.1	Developmental (Cuticle synthesis/Protein folding)
Peptidyl-prolyl cis-trans isomerase	*Haemonchus contortus*	CDJ93250	2	21.7	120.4	Developmental (Cuticle synthesis/Protein folding)
Peptidyl-prolyl cis-trans isomerase 3	*Caenorhabditis elegans*	P52011	3	42.6	162.9	Developmental (Cuticle synthesis/Protein folding)

Phosphatidylethanolamine-binding	*Caenorhabditis remanei*	XP_003115303	2	61.3	91.8	Metabolism (Lipid binding/Serine protease inhibitors)
Phosphatodylethanolamine binding	*Caenorhabditis elegans*	O16264/ F40A3.3	2	101.4	128.9	Metabolism (Lipid binding/Serine protease inhibitors)
Phosphatodylethanolamine binding	*Haemonchus contortus*	CDJ94653	3	19.2	247.2	Metabolism (Lipid binding/Serine protease inhibitors)
Polyprotein/allergen DVA-1	*Dictyocaulus viviparus*	Q24702	2	78.1	122.2	Unknown
Profilin	*Caenorhabditis elegans*	Q9XW16	6	55.7	292.8	Cytoskeletal protein (Actin binding)
Profilin	*Caenorhabditis remanei*	XP_003094677	6	32	292.8	Cytoskeletal protein (Actin binding)
Profilin	*Haemonchus contortus*	CDJ96632	2	13.2	161.1	Cytoskeletal protein (Actin binding)
Protein disulfide-isomerase 2	*Ascaris suum*	ADY47890	2	147	66.6	Metabolism (Other)
Protein disulphide isomerase	*Caenorhabditis elegans*	T34092/BG734024	2	51.3	66.6	Metabolism (Other)
Protein disulphide isomerase	*Teladorsagia circumcincta*	ABC86956	2	148.8	66.6	Metabolism (Other)
Protein NAS-28 Astacin	*Carnorhabditis elegans*	CCD65318	2	128.2	48.0	Developmental (Growth/Structural)
Protein RO5A10	*Caenorhabditis elegans*	NP_001255794	5	25.7	233.0	Unknown
Putative cytochrome c	*Haemonchus contortus*	ACG69807	5	39.3	229.4	Metabolism (Electron transport)
Putative ES protein F7	*Ostertagia ostertagi*	Q8WQ12	4	15.3	196.0	Metabolism (Lipid binding)
Putative Lipid Binding Protein	*Angiostrongylus cantonensis*	CAR63537	6	28.5	393.4	Molecular (Lipid binding)

Putative major allergen	*Angiostrongylus cantonensis*	CAR63553	5	88.3	251.9	Unknown
Putative major allergen	*Haemonchus contortus*	CDJ89445.	3	27.4	256	Unknown
Putative myoglobin	*Trichinella spiralis*	XP_003374421	5	46.1	342.1	Metabolism (Lipid binding)
Putative protein HEH-1	*Caenorhabditis elegans*	O17271	4	61.4	238.7	Metabolism (Lipid binding)
Putative pterin-4-alpha-carbinolamine	*Caenorhabditis elegans*	Q9TZH6	4	35.7	224.7	Metabolism (Amino acid)
Saposin type B	*Haemonchus contortus*	CDJ88233	2	48.6	72.4	Gut protein
Saposin-like Protein family member	*Caenorhabditis elegans*	NP_741465	2	37.3	71.3	Gut protein
Secreted protein ASP-2	*Necator americanus*	AAP41952	3	26.8	166.1	Developmental (Reproduction/Transport)
Superoxide dismutase	*Haemonchus contortus*	Q27666	2	16.6	147.2	Metabolism (Detoxification)
Superoxide dismutase extracellular	*Haemonchus contortus*	P51547	6	37.5	401.1	Metabolism (Detoxification)
Telomerase-associated protein	*Haemonchus contortus*	CDJ93215	3	15.3	109.9	Cellular (Transport)
Thioredoxin like	*Caenorhabditis elegans*	NP_001022003	2	46.4	76.6	Cytoskeletal protein
Thrombospondin	*Haemonchus contortus*	AF043121	9	171.8	468.2	Developmental (Reproduction/Growth)
Thrombospondin	*Haemonchus contortus*	AAB99830	3	33.1	120.3	Developmental (Reproduction/Growth)
Transthyretin like	*Caenorhabditis brenneri*	EGT36246	6	56.6	238.8	Transport
Transthyretin like hypothetical protein Cbre_JD14.007	*Caenorhabditis brenneri*	ACI49119	4	37.4	224.1	Transport

Transthyretin like protein	*Caenorhabditis elegans*	NP_502060	3	93.8	153.6	Transport
Transthyretin like protein	*Haemonchus contortus*	CDJ96905	2	15.7	143.2	Transport
Transthyretin-related	*Heligmosomoides polygyrus bakeri*	CCC54337	6	39.6	330.3	Transport
Unnamed protein product	*Haemonchus contortus*	CDJ92656	2	65.3	98.5	Unknown
Venom-allergen-like (VAL) protein	*Caernorhabditis brenneri*	EGT59294	441.1	9	66.6	Developmental

Appendix 3: Phage clones identified by panning 7-mer phage display libraries

L_3 somatic antigens:

Clone ID	Peptide insert	Identified in control panning	Target unrelated peptide
1	AKIDART		
2	YGFVPSW		
3	YGFVPSW		
4	NDSVSLP		
5	SAPSSKN		
6	YGFVPSW		
7	STASYTR		
8	HAIYPRH	Yes	Yes
9	KAVHPLR		
10	SPSMLQK		
11	WPTLQWA		
12	WPTLQWA		
13	HAIYPRH	Yes	
14	YGDALFA		
15	HAIYPRH	Yes	
16	GSHNPHL		
17	NGYSWTS		
18	WPTLQWA		
19	AKID*RT		
20	GSHNPHL		
21	THLPWQT		Yes
22	KLPGWSG	Yes	
23	KLPASLT		Yes
24	HAIYPRH	Yes	
25	WPTLQWA		
26	LPLTPLP		Yes
27	WPTLQWA		
28	LMRSPDY		
29	HVPLLAT		Yes
30	NTTPERS		
31	WPTLQWA		
32	WPTLQWA		
33	RLFTIPV		
34	HAIYPRH	Yes	
35	TIEQHPP		

36	HAIYPRH	Yes	
37	HAIYPRH	Yes	
38	-		
39	AKIDART		
40	YLTMPTP		Yes
41	WPTLQWA		
42	LTHPRWP		Yes
43	SPYSLYA		
44	SPSMLQK		
45	QSPDEVW		
46	MVPKWVA		
47	WGIRYPA		
48	AKIDART		
49	GRLSYPP		
50	ITSRTAS		
51	FMRSPPM		
52	LPLTPLP		Yes
53	TTSLLSD		
54	HAIYPRH	Yes	
55	KLPGWSG	Yes	
56	HAIYPRH	Yes	
57	WPTLQWA		
58	WPTLQWA		
59	STVTLQV		
60	REIHARH		
61	QHPAKTA		
62	FMRSPPM		
63	SSHTISF		Yes
64	YSIPKSS	Yes	
65	VASAGPH		
66	SAPSSKN		
67	APYQLLV		

L_3 native surface antigens:

Clone ID	Peptide insert	Identified in control panning	Target unrelated peptide
1	SLLIKPR		
2	HTPSGVA		
3	SYVSPHN		
4	GPRLMDM		
5	TLEAQTH		
6	ANTSSLP		
7	LPSHALR		
8	SGSISSS		
9	MPSLPPP		
10	ELTHLK		
11	WTPSVRP		
12	SDLLGLP		
13	WPTLQWA		
14	TSPTSFD		
15	WPTLQWA		
16	IHMQRAI		
17	SILPYPY		Yes
18	SLPTLTL		Yes
19	HAIYPRH	Yes	Yes
20	QHQLGLL		
21	QPWPTSI		
22	SWPQRTN		
23	WPTLQWA		
24	SPQMTLS		
25	KFLPVFT		
26	GWPKFTK		
27	ATLEMPP		
28	ALASVSI		
29	APPRLLY		
30	WTPSVRP		
31	TPRLQSS		
32	KLPGWSG	Yes	Yes
33	ALIPKPR		
34	ALTGIDS		
35	GTWLSR		
36	QLKTGLA		Yes
37	QPWPTSI		
38	FGHQQKM		

39	GNTPSRA		Yes
40	NPPSGRP		
41	ALTGIDS		
42	HAIYPRH	Yes	Yes
43	ALTPTPP		
44	ASTLKWA		
45	SILPYPY		Yes
46	SWPQRTN		
47	WPTLQWA		
48	GWPKFTK		
49	WTPSVRP		
50	HAIYPRH	Yes	Yes
51	QSIDMWI		
52	WPTLQWA		
53	QSPVSTQ		
54	SHVPMNP		
55	NPPSRHP		
56	KLPGWSG	Yes	Yes
57	KLPGWSG	Yes	Yes
58	THVMQTL		
59	GNTLAVQ		
60	EPLQLKM		

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